ISOFORMS OF SNARE MOLECULES AND THE USES THEREOF IN MODULATION OF CELLULAR EXOCYTOSIS

METHODS OF TREATMENT

The present invention relates to methods of treatment, in particular methods relating to treating or preventing poisoning by clostridial toxins.

Tetanus toxin (TeTx) and botulinum toxin (BoNT) are potent neurotoxins that induce paralysis through the inhibition of neurotransmitter release.

Botulism is a paralyzing disease caused by the toxin of *Clostridium botulinum* (see Cherington (1998) *Muscle Nerve* **21(6)**, 701-710 for a review). The toxin produces skeletal muscle paralysis by producing a presynaptic blockade to the release of acetylcholine. The several types of botulinum neurotoxin act at the nerve terminal. Since the discovery of the toxin about 100 years ago, five clinical forms of botulism have been described: 1) classic or foodborne botulism; 2) wound botulism; 3) infant botulism; 4) hidden botulism; 5) inadvertent botulism. A clinical pattern of descending weakness is characteristic of all five forms. Almost all human cases of botulism are caused by one of three serotypes (A, B, or E). Classic and wound botulism were the only two forms known until the last quarter of this century. Wound botulism was rare until the past decade. Now there are increasing numbers of cases of wound botulism in injecting drug users. Infant botulism, first described in 1976, is now the most frequently reported form. In infant botulism spores of *Clostridium botulinum* are ingested and germinate in the intestinal tract. Hidden botulism, the adult variant of infant botulism, occurs in adult patients who usually have an abnormality of the intestinal tract that allows colonization by *Clostridium botulinum*. Inadvertent botulism is the most recent form to be described. It occurs in patients who have been treated with injections of botulinum toxin for dystonic and other movement disorders.

Laboratory proof of botulism is established with the detection of toxin in the patient's serum, stool, or wound. The detection of *Clostridium botulinum* bacteria in the stool or wound should also be considered evidence of clinical botulism. Electrophysiologic studies can provide presumptive evidence of botulism in patients with the clinical signs of botulism. Electrophysiologic testing can be especially helpful when bioassay studies are negative. The most consistent electrophysiologic abnormality is a small evoked muscle action potential in response to a single supramaximal nerve stimulus in a clinically affected muscle. Post-tetanic facilitation can be found in some affected muscles. Single-fibre EMG studies typically reveal increased jitter and blocking, which become less marked following activation.

In humans, neuromuscular paralysis resulting from exposure to botulinum neurotoxin (BoNT) is often fatal, and survivors require a year or more to achieve full recovery [Sloop *et al* (1997) *Neurology* **49**, 189-194]. The major treatment for severe botulism is advanced medical and nursing supportive care with special attention to respiratory status.

Tetanus can result from contamination of a wound, for example a needle puncture wound, by spores of *Clostridium tetani*. The spores germinate under anaerobic conditions and toxin is released on death of the bacteria. The symptoms of tetanus are predominantly those of rigidity of voluntary muscles, including characteristic trismus (lockjaw) and violent spasms of the abdomen, back and limbs with death commonly due to asphyxiation. Vaccination with tetanus toxoid is effective and therefore tetanus is largely restricted to areas of the world in which vaccination is not widespread. The management of tetanus is reviewed, for example, in Spaeth R (1969) *JAMA* **207(2)**, 370 and in Bleck TP (1994) *J R Soc Med* **87(11)**, 719-20.

Seven homologous serotypes of BoNT, termed A-G, are produced by different *Clostridium botulinum;* each has a molecular weight of about 150 kD and consist of a

heavy and light chain (LC) linked by a disulphide bridge and non-covalent bonds. BoNTs target motor nerve endings by binding avidly to distinct ecto-acceptors, exclusively located on cholinergic presynaptic membranes, with subsequent acceptor-mediated uptake and translocation to the cytosol where they block transmitter release. This is due to the LCs being Zn^{2+}-dependent neutral endoproteases with each having a strict specificity to cleave a distinct peptide bond, in one (usually) of three proteins essential for Ca^{2+}-regulated transmitter release: SNAP-25, synaptosomal-associated protein of Mr=25 kD (BoNT/A, E, C1); syntaxin 1 (BoNT/Cl) and synaptobrevin (BoNT/B, D, F or G).

A single type of neurotoxin, tetanus toxin (TeTx) appears to be synthesised by *Clostridium tetani*. The toxin appears to be taken up by motor neurons and transported to the spinal cord where it blocks transmitter release at central inhibitory nerve terminals (4). The structure of TeTx is analogous to that of the botulinum toxins. The LC is a Zn^{2+}-dependent neutral endoprotease that cleaves synaptobrevin (Sbr) and cellubrevin (reviewed in 4). Human synaptobrevin/VAMP-2 is cleaved between Gln76 and Phe77 by TeTx (see, for example Shciavo *et al* (1992) *EMBO J* **11**, 3577).

SNAP-25, syntaxin 1 and synaptobrevin are termed SNARE (soluble NSF-attachment protein receptor, where NSF is *N*-ethylmaleimide-sensitive fusion protein) polypeptides. Poisoning with BoNT/A appears to be more severe than poisoning arising from the other botulinum toxins. SNAP-25 and syntaxin 1 are target membrane SNAREs (tSNAREs) whereas synaptobrevin is a vesicle-membrane SNARE (vSNARE). Multiple isoforms of vSNARES and tSNARES have been described (reviewed in, for example, Gonelle-Gispert *et al* (1999) *Biochem J* **339**, 159-165 and Linial (1997) *J Neurochem* **69**, 1781-1792).

Although prompt administration of neutralising antibodies can reduce the possibility of death, these are ineffective toward toxin already internalised within the motor nerves of patients displaying the symptoms of botulism. For persons at risk of botulism, for example military personnel, vaccination with botulinum toxoid is possible. However, there is growing concern about vaccination with toxoid, because of the widespread and successful use of BoNT/A in the treatment of numerous muscle movement disorders; vaccination with toxoid may render subsequent treatment with BoNT/A ineffective. Muscle movement disorders that may be treated using BoNT/A include a variety of dystonias and dysphonias – see, for example Gordon (1999) The role of botulinus toxin type A in treatment-with special reference to children. *Brain Dev* **21(3)**, 147-51. Increasing unpopularity of this prophylaxis may lead to its restricted application. In view of this, and the other above-noted factors, there is a need to design novel treatments for human clostridial poisoning, for example botulism.

One approach is to develop a small, effective and specific inhibitor of the protease for each light chain (LC), which must be amenable to targeting and delivery inside the poisoned nerve terminals (see Schmidt *et al* (1998) *FEBS Lett* **435(1)**, 61-64). Small synthetic peptide inhibitors are described, the most avid being N-acetyl-CRATKML-carboxamide, with a K_i of ~2μM.

We demonstrate that the inhibitory action of clostridial toxin on exocytosis may persist for several weeks, which may be due to a predominance of the toxin-cleaved SNARE protein over the full length functional SNARE protein. We further demonstrate that rescue of exocytotic function in clostridial toxin-poisoned cells may be achieved through supply to the cell of a polypeptide that is resistant to proteolytic cleavage by the toxin and is capable of performing the function of the target protein and/or is capable of inhibiting the proteolytic activity of the toxin. Supply to affected cells of

such a polypeptide may be used in the treatment of clostridial toxin poisoning, for example botulism. This may be done in conjunction with other treatments, for example administration of a protease inhibitor, as described above.

This novel treatment may afford relatively fast rescue of transmitter release, alleviating the symptoms when most severe and taking the patient out of the critical state. Furthermore, this treatment may pre-empt BoNT/A-induced nerve sprouting and long-term remodelling of the motor endplates (de Paiva *et al* (1999) *Proc. Natl. Acad. Sci. (USA)* **96**, 3200-3205) and may avoid the poisoning-associated extensive atrophy of the muscle fibres and negate the need for months of rehabilitation.

Further, we demonstrate that a truncated clostridial toxin target protein may inhibit exocytosis, particularly regulated exocytosis. Supply of a truncated clostridial toxin target protein or related polypeptide to a cell in which it is desirable to inhibit exocytosis may be useful in medicine, for example as an alternative to the use of BoNT/A, for example in the treatment of muscle movement disorders, or for the inhibition of exocytosis in cells to which clostridial toxins do not bind, for example adreno-chromaffin cells or insulin-secreting cells. It will be appreciated that regulated membrane fusion events (such as occur in regulated exocytosis) that have been investigated appear to require proteins homologous to SNAP-25, synaptobrevin and syntaxin 1.

A first aspect of the invention provides a method of treating a patient suffering from poisoning or at risk of poisoning by a clostridial toxin wherein a SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor) is supplied to a cell of the patient, wherein the SNARE is resistant to proteolysis by the said clostridial toxin (toxin-resistant SNARE) and/or is capable of inhibiting the clostridial toxin (toxin-inhibitory SNARE).

It is preferred that the said cell is a cell in which it is desirable to prevent or reduce inhibition of Ca^{2+}-regulated exocytosis that may be caused by contact of the said clostridial toxin with the said cell. Thus, it is preferred that the said cell is a cell in which the said clostridial toxin is capable of inhibiting Ca^{2+}-regulated exocytosis. Where the clostridial toxin is a botulinum toxin, the cell may be a cholinergic neuron. Where the clostridial toxin is a tetanus toxin, the cell may be an inhibitory neuron in the spinal cord.

It will be appreciated that by "supplied to a cell" is included over-expression of the said toxin-resistant or toxin-inhibitory SNARE in the said cell, for example from a recombinant polynucleotide capable of expressing the said toxin-resistant or toxin-inhibitory SNARE in the said cell, as discussed further below. By over-expression is included the meaning that the said toxin-resistant or toxin-inhibitory SNARE is expressed to a detectable level at least 1.5, 2, 5, 10, 20, 50, 100, 150, 200, 500, 1000 or 5000 times greater than the detectable level of the said toxin-resistant SNARE in an equivalent cell to which the toxin-resistant or toxin-inhibitory SNARE has not been supplied. It will be appreciated that if the toxin-resistant or toxin-inhibitory SNARE is not a naturally occurring SNARE then the toxin-resistant or toxin-inhibitory SNARE may not be present and/or detectable in the said equivalent cell. It may be preferred that the said toxin-resistant or toxin-inhibitory SNARE is expressed to a detectable level at least 1.5, 2, 5, 10, 20, 50, 100, 150, 200, 500, 1000 or 5000 times greater than the detectable level of an equivalent naturally occurring, non-toxin-resistant or non-toxin-inhibitory SNARE in the cell.

Typically, the patient to be treated is administered an effective amount of the said toxin-resistant or toxin-inhibitory SNARE and/or of a recombinant polynucleotide capable of expressing the said toxin-resistant or toxin-inhibitory SNARE in the said cell. By effective amount we include an amount sufficient to produce a clinically useful or significant reduction in the symptoms of poisoning by the said clostridial

toxin in the said patient suffering from poisoning, or in the said patient at risk of poisoning when exposed to the said clostridial toxin. The effective amount may produce an increase in exocytosis in the said cell.

It will be appreciated that administration of an effective amount of the said toxin-resistant or toxin-inhibitory SNARE and of a recombinant polynucleotide capable of expressing the said toxin-resistant or toxin-inhibitory SNARE in the said cell may be useful. The administered toxin-resistant or toxin-inhibitory SNARE may be able to act before toxin-resistant or toxin-inhibitory SNARE is expressed and/or transported to the appropriate part of the cell. Thus, this approach could potentially enable a more rapid recovery of exocytosis. Gene expression and transport of newly synthesised toxin-resistant or toxin-inhibitory SNARE, for example BoNT resistant SNAP-25, from the nerve cell body to the nerve synapses by rapid axonal transport may take days for humans. Synapse-targeted liposomes incorporating membrane linked toxin-resistant or toxin-inhibitory SNARE, for example SNAP-25 (linked, for example, by site specific reaction of centrally-located cysteines *via* a reactive thiol specific MPB– phosphatidyl ethanolamine (Avanti Polar Lipids Inc) may be useful in directing the administered toxin-resistant or toxin-inhibitory SNARE to the correct cellular location. Upon fusion of these liposome with the synaptic PM – membranes will merge and the toxin-resistant or toxin-inhibitory SNARE, for example SNAP-25 (some in the appropriate orientation) will be provided to the synapse.

It will be appreciated that it may be beneficial (in relation to this and further aspects of the invention) to supply more than one toxin-resistant or toxin-inhibitory SNARE to a cell of the patient. For example, it may be beneficial to supply toxin-resistant and/or toxin-inhibitory syntaxin and toxin-resistant and/or toxin-inhibitory SNAP-25 to a cell of a patient when the clostridial toxin is botulinum toxin C1. Both these SNAREs are cleaved by botulinum toxin C1. Therapy directed against botulinum toxin C1 may be particularly important in relation to fowl or domesticated animals.

A further aspect of the invention provides the use of a SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor), or of a recombinant polynucleotide capable of expressing the said SNARE in the manufacture of a medicament for the treatment of a patient suffering from poisoning or at risk of poisoning by the said clostridial toxin, wherein the SNARE is resistant to proteolysis by the said clostridial toxin (toxin-resistant SNARE) and/or is capable of inhibiting the clostridial toxin (toxin-inhibitory SNARE).

The toxin-resistant or toxin-inhibitory SNARE, as discussed further below, may be useful in combating clostridial toxin poisoning, for example botulism or tetanus.

It is preferred that the said patient is a human. Less preferably, the patient may be a non-human mammal, for example a domesticated animal, for example a rodent (for example a mouse or a rat) or domesticated mammal, for example a horse or dog. It will be appreciated that many types of live stock or domesticated animals may be susceptible to botulism. Further, it will be appreciated that the high level of sequence identity between equivalent SNAREs, for example SNAP-25s, from different animals, for example mammals, may mean that a SNARE, for example a toxin-resistant or toxin-inhibitory SNARE, that is capable of supporting exocytosis in cells of one type of animal, for example mammal, may also be capable of supporting exocytosis in cells of a different type of animal and may therefore be useful in treating both types of animal.

A further aspect of the invention provides a method of reversing the inhibition of exocytosis in a cell caused by contact of a clostridial toxin with the said cell, wherein a SNARE is supplied to the said cell not before contact of the said clostridial toxin with the said cell, wherein the SNARE is resistant to proteolysis by the said clostridial

toxin (toxin-resistant SNARE) and/or is capable of inhibiting the clostridial toxin (toxin-inhibitory SNARE). The method may be performed *in vivo* or *in vitro*.

It is preferred that the cell is a mammalian cell, more preferably a human or rodent cell, still more preferably a human cell. It is preferred that the clostridial toxin is BoNT/A, BoNT/C1 or TeTx. It may be preferred that the clostridial toxin is not BoNT/E. It is particularly preferred that the toxin-resistant SNARE is resistant to proteolysis by two or more clostridial toxins, preferably all clostridial toxins that are capable of cleaving the equivalent native non-toxin-resistant SNARE. For example, it is preferred that SNAP25 or equivalent SNARE is resistant to proteolysis by two or more of BoNT/E, BoNT/C1 and BoNT/A. It is preferred that synaptobrevin is resistant to proteolysis by two or more of BoNT/B, BoNT/D, BoNT/F or BoNT/G, and/or tetanus toxin. Thus, a single polypeptide (or polynucleotide encoding the polypeptide) may be used to treat patients suffering from clostridial poisoning before the culprit toxin/serotype has been determined.

It is further preferred that the said SNARE is supplied to the cell not before 30, 40, 50 or 60 minutes or 2, 3, 6, 12 or 18 hours or 1, 2, 5, 10, 14, 21 or 28 days or 2 or 3 months after first contact of the said clostridial toxin with the said cell. It is preferred that the cell is exposed to the toxin (ie toxin is present in the external medium surrounding the cell) for at least 30, 40, 50 or 60 minutes or 2, 3, 6, 12 or 18 hours or 1, 2, 5, 10, 14, 21 or 28 days. It is preferred that the cell is an intact cell. Thus, it is preferred that the cell is not a permeabilised cell, for example a cell permeabilised by treatment with digitonin or SLO (streptolysin O; see for example Gonelle-Gispert *et al* (1999)) or a permeabilised PC12 cell. It is preferred that the said inhibition of exocytosis is at least 60%, preferably 70, 80, 85, 90 or 95% inhibition of the exocytosis of which the cell was capable before exposure to the said clostridial toxin.

The term SNARE (soluble (*N*- ethylmaleimide-sensitive fusion protein)-attachment protein receptor) is well known to those skilled in the art, for example Gonelle-Gispert *et al* (1999) *Biochem J* **339**, 159-165 and Linial (1997) *J Neurochem* **69**, 1781-1792. SNARE polypeptides are considered to be involved in Ca^{2+}-regulated exocytosis, for example release of neurotransmitters from nerve terminals, insulin (stored in large dense-core granules) release, for example from pancreatic B cells or the HIT (hamster) or RIN (rat) insulin-secreting cell lines and evoked exocytosis from chromaffin cells. Chromaffin cells are the secretory cells of the adrenal medulla.

Examples of SNAREs include SNAP-25 (synaptosomal-associated protein of 25 kDa), syndet (or SNAP-23), the VAMP (vesicle-associated membrane protein) vSNARE sub-family (which includes VAMP-1 (synaptobrevin1), VAMP-2 (synaptobrevin2) and cellubrevin) and the syntaxin tSNARE sub-family which has more than 12 isoforms with different tissue distributions as well as different cellular localizations. Syntaxin 1a and 1b are largely neuron or neuroendocrine specific.

It will be appreciated that a SNARE may be capable of forming a complex with the NSF (*N*-ethylmaleimide-sensitive fusion protein) or a SNAP (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein). SNAREs may contain homologous domains that form coiled-structures that may mediate interaction between SNAREs, as known to those skilled in the art.

SNAP-25 is present in two isoforms (a and b) in neurons (Bark (1993) *J Mol Biol* **233**, 67-76; Bark & Wilson (1994) *Gene* **139**, 291-292). The isoforms appear to arise from alternative splicing of two divergent versions of exon 5 and differ by nine amino acids and the spacing of four cysteine residues which are palmitoylated and participate in the membrane association of SNAP-25. Both forms appear to be able to support insulin secretion in HIT cells (Gonelle-Gispert *et al* (1999) *Biochem J* **339**, 159-165). Zhao *et al* (1994) *Gene* **145(2)**, 313-314 report that human SNAP-25 may have an

identical amino acid sequence to mouse SNAP-25. Human and mouse SNAP-25b are 95.6% identical at the amino acid level with a 100% homology at the relevant C-terminus. Non-identical residues are located towards the N-terminus and have analogous amino acid substitutions. There are six amino acid changes of which 5 are conservative. The sequences of human SNAP-25 isoforms a and b are given in Figure 7.

SNAP-23 (also termed syndet) is a homologue of SNAP-25 that appears to be ubiquitously expressed and has approximately 60% amino acid identity with SNAP-25 (human SNAP-23: Ravichandran *et al* (1996) *J Biol Chem* **271**, 13300-13303; mouse SNAP-23: Araki *et al* (1997) *Biochem Biophys Res Comm* **234**, 257-262; Wang *et al* (1997) *J Cell Sci* **110**, 505-513). SNAP-23 appears to be able to perform the function of SNAP-25 in insulin secretion when overexpressed (Sadoul *et al* (1997) *J Cell Biol* **128**, 1019-1028).

SNAP-25 is cleaved by BoNT/A between Gln197 and Arg198 of full length SNAP-25. It is cleaved by BoNT/C between Arg198 and Ala199 of full length SNAP-25 and by BoNT/E between Arg180 and Ile181. Human SNAP-23 does not appear to be cleaved by BoNT/A, BoNT/C or BoNT/E *in vitro*. Rat SNAP-23 appears to be cleaved by BoNT/E and to a limited extent by BoNT/A *in vitro*. (see, for example, Vaidyanathan *et al* (1999) *J Neurochem* **72**, 327-337 and Figure 7).

Syntaxin 1 is cleaved by BoNT/C1 and synaptobrevin (Sbr) by BoNT/B, /D, /F, /G and TeTx [reviewed by (4)]. Human VAMP-2 (synaptobrevin2) is cleaved between Gln76 and Phe77 by TeTx (see, for example Shiavo *et al* (1992) *EMBO J* **11**, 3577). Cellubrevin is also cleaved by TeTx.

Galli *et al* (1998) *Mol Biol Cell* **9(6)**, 1437-1448 reports that syntaxin 3, SNAP-23 and a tetanus neurotoxin-insensitive VAMP (TI-VAMP) are insensitive to clostridial

neurotoxins. As mentioned in Gonelle- Gispert *et al* (1999), many isoforms (more than 10) of syntaxin exist. The susceptibilities of isoforms 1 - 5 to BoNT/C1 cleavage have been assessed and it appears that only 1, 2 and 3 are sensitive. In addition, within each group many minor variants have also been reported. It will be appreciated that the sensitivities of each variant to cleavage may be different.

It will be appreciated that the toxin-resistant SNARE is typically a non-naturally occurring, ie synthetic, protease-resistant variant of a naturally occurring SNARE that is capable of being cleaved by the said clostridial toxin. However, the toxin-resistant SNARE may be a naturally occurring toxin-resistant SNARE.

It is preferred that the said "SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor) that is resistant to proteolysis by the said clostridial toxin" (referred to herein as a toxin-resistant SNARE) or toxin-inhibitory SNARE is capable of performing substantially the equivalent function to a SNARE (present in the said cell) that is capable of being cleaved in the said cell by the said clostridial toxin (or is not a toxin-inhibitory SNARE, as appropriate). Still more preferably, the toxin-resistant or toxin-inhibitory SNARE is capable of performing substantially the equivalent function to a SNARE that is capable of being cleaved by the said clostridial toxin (and is not a toxin-inhibitory SNARE, as appropriate) and thereby rendered less able to perform its normal function (for example, in Ca^{2+}-mediated exocytosis). Thus, it is preferred that the toxin-resistant or toxin-inhibitory SNARE has substantially the same capability to participate in/support Ca^{2+}-mediated exocytosis as the said SNARE present in the cell that is capable of being cleaved in the said cell by the said clostridial toxin (or is not a toxin-inhibitory SNARE, as appropriate).

Thus, for example, it is considered that in cells affected by BoNT/A, BoNT/C1 or BoNT/E, SNAP-25 is cleaved, the cleaved products being unable to perform the role

of full length SNAP-25 in Ca^{2+}-mediated exocytosis. It is therefore preferred that when the said clostridial toxin is BoNT/A, BoNT/C1 or BoNT/E that the toxin-resistant SNARE is capable of performing the function of full length SNAP-25, for example in Ca^{2+}-mediated exocytosis. The said toxin-resistant SNARE that is capable of performing the function of full length SNAP-25 may be, for example, SNAP-23 (preferably human SNAP-23) or another naturally occurring SNAP-25 or SNAP-23 homologue that is resistant to cleavage by BoNT/A, BoNT/C1 and/or BoNT/E, as appropriate. It is preferred that such a naturally occurring toxin-resistant SNARE, for example SNAP-25 homologue is a mammalian homologue, preferably a human homologue, but it will be appreciated that it may be, for example, a lower eukaryotic homologue, for example yeast, *Drosophila* or *Torpedo* homologue. Alternatively, the said toxin-resistant SNARE may be a variant (which may not be naturally occuring) of SNAP-25 that is resistant to BoNT/A, BoNT/C1 and/or BoNT/E (as appropriate) and is capable of performing the function of full-length SNAP-25.

It will be appreciated full length SNAP-25 may have functions other than involvement in Ca^{2+}-mediated exocytosis, for example in membrane fusion events. For example, a developmental form of SNAP-25 may be required for growth cone elongation. SNAP-25's interaction with other synaptic proteins is known, for example-voltage dependent Ca^{2+} channels (Wiser *et al.*, 1996); this may serve to locate synaptic vesicles close to the point of Ca^{2+} entry enabling rapid neurotransmission. Synaptotagmin, a putative Ca^{2+}-trigger for neurotransmitter release also associates with SNAP-25 (Schiavo *et al.*, 1997).

Examples of such variants of SNAP-25 that are resistant to cleavage by BoNT/A are discussed in Example 1 and include variants in which the residue equivalent to residue 197 and/or the residue equivalent to residue 198 of full length SNAP-25 (for example full length mouse SNAP-25) are replaced by a residue other than Gln or a residue other than Arg, respectively. In particular the residue equivalent to residue Q197 of

full length human SNAP-25 may be Q or may be replaced, for example by A, K or W and the residue equivalent to R198 of full length human SNAP-25 may be replaced by a residue other than R, for example A, S, E, D, T, K, H or W. Such variants mutated at the residue equivalent to R198 of full length human SNAP-25, for example to A, S, T, D or E are resistant to cleavage by BoNT/C1 (see Vaidyanathan *et al* (1999) *J Neurochem* **72**, 327-337). Replacement of the residue equivalent to I181 of full length human SNAP-25, for example by F, renders the variant resistant to BoNT/E, as discussed further below.

Syntaxin 1A/B may be rendered resistant to BoNT/C1 by replacement of the residue equivalent to L235 of full length human syntaxin 1A/B, for example by A. Synaptobrevin may be rendered resistant to BoNT/B by replacement of the residue equivalent to F77 of full length human synaptobrevin by, for example, V (Shone & Roberts (1994) *Eur J Biochem* **225**, 263-270). Similarly, mutations may be made at the sites of cleavage of BoNT/D, BoNT/F and/or BoNT/G.

Methods of determining whether a polypeptide, for example a toxin-resistant (or toxin-inhibitory) SNARE, is capable of performing the function of a SNARE that is cleaved by the said clostridial toxin (or is not a toxin-inhibitory SNARE, as appropriate) will be well known to those skilled in the art and include methods as described or reviewed in Example 1. Thus, the ability of the said toxin-resistant or toxin-inhibitory SNARE to support evoked exocytosis in toxin-treated cells may be measured, for example using known techniques for measuring secretion, for example as described in Example 1. It is preferred that the toxin-resistant SNARE is capable of performing the function of a polypeptide that is capable of being cleaved by the said clostridial toxin, for example is capable of supporting evoked exocytosis in toxin-treated cells, to at least (in order of preference) 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the level (for example of evoked exocytosis) achieved by

the said polypeptide that is capable of being cleaved, in cells that have not been treated with toxin.

It will be appreciated that experiments have demonstrated that overexpression of SNAP-25 as compared to native SNAP-25 may not increase the level of exocytosis. Therefore, the finite amount of syntaxin 1 and Sbr present (with which SNAP-25 interacts during exocytosis) may define the upper limit of exocytosis. Thus, over-expression of a toxin-resistant SNARE, for example syntaxin 1, synaptobrevin and/or SNAP-25, or variants thereof, for example in non-neuronal cells may increase the absolute levels of exocytosis and accentuate a secretory mechanism allowing more than 100% of the level of exocytosis of which the untreated cell (ie untreated with toxin or with toxin-resistant SNARE) was capable.

Examples of variants of SNAP-25 that are resistant to BoNT/E include variants in which the residue equivalent to residue 180 and/or the residue equivalent to residue 181 of full length SNAP-25 (for example full length mouse SNAP-25) are replaced by a residue other than Arg or a residue other than Ile, respectively. For example, Ile181 may be replaced by Phe, Gly, Ser or Asn. Replacement by Val may also result in a small increase in resistance to BoNT/E cleavage (Vaidyanathan *et al* (1999) *J Neurochem* 72, 327-337). Arg176, Asp179 and/or Met182 may further or alternatively be mutated, for example to Pro176, Lys179 and/or Thr182 (see Gonelle-Gispert *et al* (1999). Gonelle-Gispert *et al* (1999) demonstrated that BoNT/E resistant SNAP-25s incorporating alterations at positions 180 and 181 could efficiently function in evoked insulin secretion.

It is preferred that the variant of SNAP-25 is resistant to cleavage by BoNT/A, BoNT/C1 and BoNT/E, for example is mutated at one or more position discussed above as important for BoNT/E cleavage, in addition to one or more position discussed above as important for BoNT/A and/or BoNT/E cleavage.

Whilst it is preferred that the toxin-resistant SNARE is capable of performing the function of a SNARE that is capable of being cleaved by the said clostridial toxin, it will be appreciated that this may not be essential. The said toxin-resistant SNARE may, for example, alternatively be a SNARE that is capable of interacting with the said SNARE that is capable of being cleaved by the said clostridial toxin (either before or after such cleavage). The said toxin-resistant SNARE may be a naturally occurring SNARE. Thus, when the said clostridial toxin is BoNT/A, the toxin-resistant SNARE may be, for example, syntaxin 1 or synaptobrevin. It will be appreciated that full length SNAP-25 may be capable of interacting with syntaxin 1 and synaptobrevin (or homologues thereof) to form a ternary complex required for exocytosis. SNAP-25 cleaved by BoNT/A or BoNT/E may be capable of interacting with syntaxin 1 and synaptobrevin but the complex formed may not be capable of supporting exocytosis, as described in Example 1. Supply (for example, over-expression) of syntaxin 1 and/or synaptobrevin (or a homologue thereof) may help to overcome the inhibition of exocytosis caused by cleavage of SNAP-25 by providing additional syntaxin 1 and/or synaptobrevin for any uncleaved SNAP-25 to bind. This supply may increase exocytosis to a level higher than that of which the cell was capable before clostridial poisioning or said supply. Whilst cleavage of syntaxinl or Sbr by the appropriate Clostridial neurotoxin is believed to inhibit exocytosis through the truncation of the domain required for ternary complex formation (12,13), BoNT/A or E truncated SNAP-25 inhibits release without preventing ternary complex formation (12). As shown in Example 1, residues 1 to 202 of SNAP-25, for example, are sufficient to support exocytosis whereas C-terminally shorter forms (1 to 197, 198, 199 or 200 of SNAP-25) inhibit exocytosis.

By a "SNARE that is resistant to proteolysis by the said clostridial toxin (toxin-resistant SNARE)" is included the meaning that (1) the function or activity of the SNARE is unaltered by cleavage by the said clostridial toxin and/or (2) the toxin-

resistant SNARE is cleaved by the said clostridial toxin to a lesser extent than a SNARE that is cleaved by the said clostridial toxin (for example SNAP-25 for BoNT/A, BoNT/C or BoNT/E; synaptobrevin for BoNT/B, D, F or G and TeTx; syntaxin for BoNT/C). Thus, in (1), the ability of the toxin-resistant SNARE to bind to other cellular components so as to allow exocytosis may be effectively unaltered by cleavage by the said clostridial toxin. In (2), by cleaved to a lesser extent is included the meaning that at least about 1.2, 1.5, 2, 4, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, 30000 or 40000 more of the said clostridial toxin is required to cleave 50% of the said toxin resistant SNARE than is required to cleave 50% of full length human SNAP-25 (for BoNT/A, BoNT/C or BoNT/E) or full length human synaptobrevin (for BoNT/B, D, F or G and TeTx) or full length human syntaxin 1 (for BoNT/C) under the same conditions, for example the conditions employed in the experiments summarised in Table 1 and described in Example 1. Methods of determining the amount of clostridial toxin required to cleave 50% of a polypeptide will be well known to those skilled in the art and are described, for example, in Gonelle-Gispert *et al* (1999), Vaidyanathan *et al* (1999) and Example 1. Table 1 shows the results of representative experiments performed using the preferred ELISA-based assay described in Example 1 on variants of SNAP-25 described in Example 1. It will be appreciated that more BoNT/A resistant SNAP-25s may be generated than those described in Example 1, for example by additional alteration of one or more of residues 1 to 206, for example 181 to 206. Careful replacement of multiple amino acids may allow maximisation of exocytotic functioning and minimisation of susceptibility of BoNT/A protease. Also, deletion of residues 202-206 from SNAP-25 makes the polypeptide a very poor substrate (Schmidt and Bostain, 1997 and our unpublished results) but functions efficiently in exocytosis. Mixing of different alterations may give better toxin resistance. A highly BoNT/A resistant SNARE, for example SNAP-25 may be useful, for example, in avoiding scission during the course of or following weeks of toxin exposure in neurons. Of additional importance may be ensuring that the altered SNAP-25 or other SNARE will

be expressed adequately and evade cellular turnover for a sufficient period (a period dependent upon type of expression vector and whether a polypeptide or polynucleotide is introduced into the cell) and function as well or more efficiently, than wild-type polypeptide.

It will be appreciated that it may be desirable to use a SNARE, for example SNAP-25, with the fewest amino acid sequence modifications necessary to provide a level of toxin resistance adequate to achieve the desired effect.

It will be appreciated that particularly if an inhibitor, for example N-acetyl-CRATKML-carboxamide (see Schmidt & Bostian (1998)), of the said clostridial toxin is supplied to the cell in addition to the said toxin-resistant SNARE that it may be adequate to supply a toxin-resistant SNARE that has low toxin-resistance to the cell ; for example, the toxin-resistant SNARE may require no more than about 1.2, 1.5, 2, 4, 5, 10, 20, 50, 100, 200 or 500 (in order of preference) more of the said clostridial toxin to cleave 50% of the said toxin resistant SNARE than is required to cleave 50% of full length human SNAP-25 (for BoNT/A, BoNT/C or BoNT/E) or full length human synaptobrevin (for BoNT/B, D, F or G and TeTx) or full length human syntaxin 1 (for BoNT/C) under the same conditions, as discussed above.

A SNARE which is capable of inhibiting the clostridial toxin (toxin-inhibitory SNARE) may usefully be supplied to the cell as indicated above, ie may be used as an alternative or in addition to a toxin-resistant SNARE in previous aspects of the invention. The toxin-inhibitory SNARE may be capable of inhibiting the protease activity of the clostridial toxin following cleavage of the SNARE by the toxin. The intact SNARE or cleavage product may be an inhibitor because it may interact with high affinity with the toxin, due to its similarity with the normal toxin substrate. A SNARE in which the residue immediately N-terminal to the toxin cleavage site is replaced by a cysteine residue (for example SNAP-25 Q197C, in relation to BoNT/A)

may, when cleaved by the clostridial toxin (for example BoNT/A in the case of SNAP-25 Q197C), produce a potent inhibitor, due to the resultant mobile thiol chelating the essential Zn^{2+} at the toxin's protease active site, in addition to the high affinity of interaction of the remainder of the SNARE with the toxin. The toxin-inhibitory SNARE is preferably capable of functioning in exocytosis at least when uncleaved by the clostridial toxin. The toxin-inhibitory SNARE may be more toxin-resistant than the native SNARE (ie may also be a toxin-resistant SNARE), but is preferably cleaved (if necessary) to an extent sufficient to cause effective inhibition of the clostridial toxin. The toxin-inhibitory SNARE may be administered in place of or in addition to another toxin-resistant SNARE.

The toxin-resistant SNARE or toxin-inhibitory SNARE may be a variant, fragment, derivative or fusion of a naturally occurring SNARE with the required or preferred properties as set out above.

By "variants" of a polypeptide, for example of SNAP-25, syntaxin 1 or synaptobrevin, we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially alter the activity of the said polypeptide, for example the ability of the SNAP-25, syntaxin 1 or synaptobrevin to participate in a ternary complex comprising SNAP-25, syntaxin 1 or synaptobrevin (or homologues thereof) which is capable of supporting exocytosis, as described above, in Example 1 and below.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

It is particularly preferred if the SNAP-25 variant has an amino acid sequence which has at least 45% identity with the amino acid sequence of mouse SNAP-25b, for

example the amino acid sequence of mouse SNAP-25b as given in Bark & Wilson (1994) *Gene* **139**, 291-292 or human SNAP-25b as shown in Figure 8, more preferably at least 50%, 55%, 60%, 65%, 70%, still more preferably at least 75%, yet still more preferably at least 80%, in further preference at least 85%, in still further preference at least 90% and most preferably at least 95% or 97% identity with the amino acid sequence defined above.

Chen *et al* (1999) *Cell* **97(2)**, 165-74 reports that a 65 aa C-terminal fragment of SNAP-25 (S25-C) may be capable of supporting exocytosis. Mutations along the hydrophobic face of the S25-C helix result in SNARE complexes with different thermostabilities, and these mutants rescue exocytosis to different extents.

Thus, a fragment of a variant of SNAP-25 that is not capable of being cleaved by a clostridial toxin, for example BoNT/A, BoNT/C1 and/or BoNT/E, as discussed above, that comprises or consists of amino acid residues equivalent to the 65 C-terminal amino acids of SNAP-25 (or the said 65 C-terminal amino acids with the exception of any or all of residues 203 to 206) may be a toxin-resistant SNARE that may be useful in the methods of the invention.

Sutton *et al* (1998) *Nature* **395(6700)**, 347-53 reports the crystal structure of a SNARE complex containing syntaxin-1 A, synaptobrevin-II and SNAP-25B. The structure reveals a highly twisted and parallel four-helix bundle. Conserved leucine-zipper-like layers are found at the centre of the synaptic fusion complex. Embedded within these leucine-zipper layers is an ionic layer consisting of an arginine and three glutamine residues contributed from each of the four alpha-helices. These residues are highly conserved across the entire SNARE family. The regions flanking the leucine-zipper-like layers contain a hydrophobic core similar to that of more general four-helix-bundle proteins. The surface of the synaptic fusion complex is highly grooved and possesses distinct hydrophilic, hydrophobic and charged regions. These

characteristics may be important for membrane fusion and for the binding of regulatory factors affecting neurotransmission; thus, it may be important to conserve some or all of these features and/or those discussed in Chen *et al* (1999) in a SNARE, for example a toxin-resistant SNARE or toxin-inhibitory SNARE, useful in the methods of the invention.

Further papers commenting on important regions and residues in SNAP-25 include Yavashi *et al* (1994) *EMBO J* **13**, 5051-5061; Chapman *et al* (1994) *J Biol. Chem.* 27427-27432; Fasshauer *et al* (1997) *J Biol Chem.* **272(44)**, 28036 and Fasshauer *et al* (1997) *J Biol Chem.* **272(7)**, 4582-90. The Cterminal residue 201 is essential (see Example 1).

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it will be appreciated that percent identity is calculated in relation to polypeptides whose sequence has been aligned optimally.

The alignment may alternatively be carried out using the Clustal W program (Thompson *et al* (1994) *Nucl Acid Res* **22**, 4673-4680). The parameters used may be as follows:

Fast pairwise alignment parameters: K-tuple(word) size; 1, window size; 5, gap penalty; 3, number of top diagonals; 5. Scoring method: x percent.

Multiple alignment parameters: gap open penalty; 10, gap extension penalty; 0.05.

Scoring matrix: BLOSUM.

"Variations" of the polypeptide also include a polypeptide in which relatively short stretches (for example 5 to 20 amino acids) have a high degree of homology (at least 80% and preferably at least 90 or 95%) with equivalent stretches of the polypeptide even though the overall homology between the two polypeptides may be much less.

This is because important active or binding sites may be shared even when the general architecture of the protein is different, as indicated above.

It is preferred that the SNARE polypeptide, for example SNAP-25, syntaxin 1 or synaptobrevin polypeptide is a polypeptide which consists of the amino acid sequence of the SNAP-25 polypeptide given in Bark & Wilson (1994) *Gene* **139**, 291-292), the syntaxin 1 sequence given in Bennett *et al* (1992) Syntaxin - A Synaptic Protein Implicated in Docking of Synaptic Vesicles at Presynaptic Active Zones, *Science* **257**, 255-259 or synaptobrevin sequence given in Elferink *et al* (1989) *J Biol Chem* **264**, 11061-11064 or Trimble *et al* (1988) VAMP-1: A synaptic vesicle-associated integral membrane protein. *Proc Natl Acad Sci USA* **85**, 4539-4542 or naturally occurring allelic variants, fragments or fusions or fusions of fragments thereof.

As discussed above, preferred variants of SNAP-25 are indicated in Example 1 and Table 1. A variant of SNAP-25 wherein the residue equivalent to Q197 of full length mouse SNAP-25b is replaced by A and the residue equivalent to R198 of full length mouse SNAP-25b is unmutated may be less preferred. A variant in which the residue equivalent to Q197 of full length mouse SNAP-25b is replaced by A and the residue equivalent to R198 of full length mouse SNAP-25b is replaced by K (or E or T) may be particularly preferred. A variant in which the residue equivalent to Q197 of full length mouse SNAP-25b is replaced by C may be particularly preferred as a toxin-inhibitory SNARE. A fragment of SNAP-25 consisting of the amino acid sequence of residues 1 to 202 of mouse SNAP-25b wherein the residue equivalent to residue Q197 is replaced by A and R198 is replaced by K (or E or T) (or a fusion of the said fragment which does not comprise residues 203 to 206 of full length mouse SNAP-25b fused to the C-terminus of the residues equivalent to residues 1 to 202 of full length mouse SNAP-25b) may be particularly preferred. SNAP-25(1 to 201), for example, incorporating the R198T mutation can efficiently support exocytosis and may be more resistant to cleavage by BoNT/A that the full length polypeptide. A

fragment of the preferred variants consisting of residues equivalent to residues about 130, 140, 150 (preferably about 140) to 202, 203, 204, 205 or 206 may also be preferred. C-terminal fragments of SNAP-25 small than 65 residues may also suffice as it appears that residues 157 to 206 may be responsible for SNAP-25s C-terminal helix formation. However, it will be appreciated that C-terminal fragments may be less effective at rescuing cells from toxin poisoning, for example BoNT/A poisoning, because such fragments may be less able to displace the SNAP-25_A protein from the 4-helical coil complex than a longer SNAP-25 fragment.

It may be preferred that a toxin-resistant SNARE polypeptide or toxin-inhibitory SNARE that is a SNAP-25 variant (including a SNAP-25 homologue) is palmitoylated (for example through thioester linkage at one or more, preferably 3 or more of the four centrally-located cysteines). Other local membrane-linking mechanisms may be employed, as well known to those skilled in the art and as discussed below.

It is preferred that the toxin-resistant or toxin-inhibitory SNARE polypeptide is a polypeptide that is capable of supporting exocytosis, for example in adreno-chromaffin cells, as discussed further in Example 1. Thus, it is preferred that if the toxin-resistant or toxin-inhibitory SNARE polypeptide is a variant, fragment, fusion or derivative of a tSNARE polypeptide that it is able to interact with a vSNARE polypeptide. If the toxin-resistant or toxin-inhibitory SNARE polypeptide is a variant, fragment, fusion or derivative of a vSNARE polypeptide, it is preferred that it is able to interact with a tSNARE polypeptide. Suitable methods of detecting such interactions include methods of measuring exocytosis in the presence of the toxin-resistant SNARE, as discussed in Example 1, yeast two-hybrid interactions, co-purification, ELISA and co-immunoprecipitation methods, as known to those skilled in the art.

It is preferred that the said toxin-resistant or toxin-inhibitory SNARE is more than 190, 180, 170, 150, 100, 80, 70, 50, 40 or 30 amino acids in length. It is further preferred that the said toxin-resistant or toxin-inhibitory SNARE is less than 500, 400, 300, 250, 210, 208, 206, 202, 198, 180, 150 amino acids in length or any combination of these maximum and minimum lengths. It is particularly preferred that the toxin-resistant or toxin-inhibitory SNARE is between 150 and about 210 amino acids in length; in further preference the toxin-resistant or toxin-inhibitory SNARE is between about 201 or 202 and about 206 amino acids in length.

By "residue equivalent to" a particular residue, for example the residue Arg198 of full-length SNAP-25, for example mouse or human SNAP-25, is included the meaning that the amino acid residue occupies a position in the native two or three dimensional structure of a polypeptide, for example a SNAP-25 homologue or variant, corresponding to the position occupied by the said particular residue, for example Arg198, in the native two or three dimensional structure of full-length SNAP-25. It will be appreciated that Arg198 of full-length SNAP-25 is located towards the C-terminus of the polypeptide.

The residue equivalent to a particular residue, for example the residue Arg198 of full-length SNAP-25, may be identified by alignment of the sequence of the polypeptide with that of full-length SNAP-25 in such a way as to maximise the match between the sequences. The alignment may be carried out by visual inspection and/or by the use of suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, which will also allow the percent identity of the polypeptides to be calculated. The Align program (Pearson (1994) in: Methods in Molecular Biology, Computer Analysis of Sequence Data, Part II (Griffin, AM and Griffin, HG eds) pp 365-389, Humana Press, Clifton). Thus, residues identified in this manner are also "equivalent residues".

It will be appreciated that in the case of truncated forms of SNAP-25 or in forms where simple replacements of amino acids have occurred it is facile to identify the "equivalent residue".

The sequence for human SNAP-25 is given in, for example Zhao *et al* (1994) *Gene* **145(2)**, 313-314. The sequences for mouse SNAP-25a and SNAP-25b isoforms are given in Bark (1993) *J Mol Biol* **233**, 67-76 and Bark & Wilson (1994) *Gene* **139**, 291-292.

The three-letter and one-letter amino acid code of the IUPAC-IUB Biochemical Nomenclature Commission is used herein. The sequence of polypeptides are given N-terminal to C-terminal as is conventional. In particular, Xaa represents any amino acid. It is preferred that the amino acids are L-amino acids, in particular it is preferred that the amino acid residues immediately flanking (such as those within 10 to 20 residues) of the clostridial toxin cleavage site consists of L-amino acid residues but they may be D-amino acid residues.

The patient may have or be at risk of botulism or tetanus. The methods of treatment of the invention are particularly suited when the patient has or is at risk of botulism, in particular botulism caused by, or by a strain producing, BoNT/A, BoNT/E, BoNT/C1, BoNT/D, BoNT/F or BoNT/G, preferably BoNT/A, BoNT/E, or BoNT/C1, still more preferably BoNT/A. It is further preferred that the patient has botulism. Thus, it is preferred that the toxin-resistant SNARE is supplied to the cells of the patient after poisoning with a clostridial toxin, for example a botulinum toxin, has occurred. Types of botulism and methods of diagnosing botulism are known to those skilled in the art and are summarised above. It will be appreciated that the patient may be an infant, for example an infant with the symptoms of a "floppy baby", that has been diagnosed as having botulism, as described above and, for example, in Greve *et al* (1993) *Monatsschr Kinderheilkd* **141(1)**, 33-35; Midura

(1979) *Rev Infect Dis* **1(4)**, 652-655; Puig de Centorbi (1998) *Zentralbl Bakteriol* **287(1-2)**, 61-6; Pickett (1982) *Muscle Nerve* **5(9S)**, S26-27.

It will be appreciated that the method of diagnosing botulism preferably allows the type of botulinum toxin that is responsible for the poisoning to be determined. Thus, it will be appreciated that the method of treatment of the invention described above may further comprise the steps of determining the type of the said clostridial, for example botulinum, toxin from which the patient is suffering and of selecting an appropriate SNARE (or SNAREs) that is/are resistant to proteolysis by the determined clostridial toxin for use in the treatment. Similarly, in the medicament –related use of the invention described above, the type of the clostridial toxin from which the patient is suffering from or at risk of poisoning by may be determined.

As noted above, the selected SNARE or SNAREs may be resistant to cleavage by more than one clostridial toxin. This may further speed up and simplify treatment by reducing the need to determine the responsible clostridial toxin before commencing treatment.

Methods of determining the type of botulinum toxin affecting a patient are well known to those skilled in the art and include antibody and nucleic acid (for example, PCR) based assays. Toxin may be identified, for example, from stool specimens, as reviewed, for example, in Pickett (1982) *Muscle Nerve* **5(9S)**, S26-27 and Cherington (1998) *Muscle Nerve* **21(6)**, 701-710.

A further aspect of the invention provides a kit of parts comprising (1) means for determining the type of clostridial, for example botulinum, toxin from which a patient is suffering or means for determining that a patient is suffering from a particular type of clostridal, for example botulinum, toxin and (2) a toxin-resistant or toxin-inhibitory SNARE or recombinant polynucleotide capable of expressing said toxin-resistant or

toxin-inhibitory SNARE. The said toxin- resistant or toxin-inhibitory SNARE may be resistant to the said particular type of clostridial, for example botulinum, toxin. It is preferred that the said clostridial toxin is BoNT/A, E or C, more preferably BoNT/A.

The toxin-resistant or toxin-inhibitory SNARE alternatively may be supplied to the cells of a patient at risk of botulism or tetanus, preferably botulism. Military personnel, for example may be patients at risk of botulism. Botulism caused by BoNT/A toxin may be more severe than that caused by other botulinum toxins; therefore it may be particularly beneficial to supply a toxin-resistant or toxin-inhibitory SNARE that is appropriate for the treatment of botulism caused by BoNT/A, as discussed above, for example a toxin-resistant or toxin-inhibitory variant of SNAP-25 that is resistant/inhibitory to proteolysis by BoNT/A (and optionally also BoNT/C and BoNT/E, as discussed above and in Example 1.

A further aspect of the invention provides a kit of parts comprising (1) a toxin-resistant (or less preferably toxin-inhibitory SNARE) or recombinant polynucleotide capable of expressing said toxin-resistant (or toxin-inhibitory) SNARE and (2) an inhibitor of the (or a) clostridial, for example botulinum, toxin to which the said toxin-resistant SNARE is resistant. Thus, the inhibitor may inhibit BoNT/A and the toxin-resistant SNARE may be resistant to BoNT/A; it may be a BoNT/A resistant SNAP-25, as described above and in Example 1. The inhibitor may be a toxin-inhibitory SNARE or recombinant polynucleotide capable of expressing said toxin-inhibitory SNARE. The kit may further comprise means for determining the type of clostridial, for example botulinum, toxin from which a patient is suffering or means for determining that a patient is suffering from a particular type of clostridal, for example botulinum, toxin, as described above.

We demonstrate in Example 1 that a fragment of a SNARE generated by cleavage by a clostridial toxin is effective in blocking exocytosis from adrenochromaffin cells. We conclude that such a fragment, for example, may be useful in inhibiting exocytosis in cells in a patient when supplied to the appropriate cells in a patient, for example by overexpression of the fragment, for example, in the appropriate cells. This method of inhibiting exocytosis may be useful as an alternative to inhibition of exocytosis by the administration of a clostridial toxin to cells susceptible to such inhibition by a clostridial toxin. Alternatively, it may be useful in inhibiting exocytosis in cells that are not susceptible to clostridial toxin *in vivo*.

Thus, a further aspect of the invention provides a method of inhibiting *in vivo* SNARE-dependent exocytosis from a cell capable of performing SNARE-dependent exocytosis wherein a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (termed inhibitory SNARE or, for further clarity exocytosis-inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is supplied to the said cell *in vivo*.

A further aspect of the invention provides a method of treating a patient in need of inhibition of SNARE-dependent exocytosis from a cell capable of performing SNARE-dependent exocytosis wherein a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is supplied to the said cell of the patient. It will be appreciated that by supply is included the expression of the said inhibitory SNARE in the said cell. The method may comprise administering to the patient an effective amount of (1) a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis or (2) a recombinant polynucleotide capable of expressing a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that

is capable of inhibiting SNARE- dependent exocytosis to the said patient. By effective amount is included the meaning of an amount effective in inhibiting SNARE-mediated exocytosis in the said cell of the patient to a clinically significant or beneficial extent.

A further aspect of the invention provides the use of (1) a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis or (2) a recombinant polynucleotide capable of expressing a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis in the manufacture of a medicament for the treatment of a patient in need of inhibition of SNARE-dependent exocytosis from a cell capable of performing SNARE-dependent exocytosis.

The said fragment of a SNARE may be a fragment derivable by cleavage of the said SNARE by a clostridial toxin, for example BoNT/A or BoNT/E.

The said inhibitory SNARE may be a fragment of SNAP-25, synaptobrevin or syntaxin 1, which terms are defined above. It is preferred that it is a fragment of SNAP-25, still more preferably a fragment of SNAP-25 derivable by cleavage of SNAP-25 by BoNT/A, for example derivable by cleavage of SNAP-25 or a variant thereof by BoNT/A between residues 197 and 198 of full length SNAP-25. It will be appreciated that by "derivable" is included the meaning of intellectually derivable; thus, the fragment may be synthesised, for example using techniques of molecular biology or synthetic peptide synthesis, without need for cleavage by a clostridial toxin, for example BoNT/A. It is particularly preferred that the said inhibitory SNARE is a fragment of SNAP-25 (or a variant thereof) wherein residues corresponding to residues 198 to 206 of full length mouse SNAP-25 are not present. Thus, the

fragment may consist of residues identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, 160, 170 or 180, preferably between 1 and about 140, to 197 of full length SNAP-25 or a variant thereof. Alternatively, the fragment may consist of residues identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, 160, 170 or 180, preferably between 1 and about 140, to 198, 199, 200 or 201 of full length SNAP-25 or a variant thereof. Such fragments of SNAP-25 may be capable of inhibiting SNARE-dependent exocytosis, as described in Example 1. The fragment may consist of residues equivalent to or identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, to 180 of full length SNAP-25 (SNAP-25(1-180)); such a fragment may be derivable by cleavage of SNAP-25 by BoNT/E. Such a fragment may be capable of inhibiting SNARE-dependent exocytosis, as described in Example 1, but may not be as potent or as persistent an inhibitor of exocytosis as SNAP-25(1-197). Methods of determining that a polypeptide is capable of inhibiting SNARE-dependent exocytosis are described, for example, in Example 1 and in Huang *et al* (1998) *Mol Endocrinol* 12(7), 1060-1070.

Alternatively, Ferrer-Mental *et al.*, 1998 have shown that polypeptides encompassing the last 20 or 26 C terminal residues of SNAP-25, blocked evoked secretion from permeabilised chromaffin cells. As the 65-mer SNAP-25 C terminal fragment can operate in exocytosis and a polypeptide of last 26 residues blocks exocytosis, the largest inhibitory C-terminal peptide must lie between these two extremes. Potentially, a larger peptide may inhibit secretion better than the 26-mer C-peptide (i.e. lower IC_{50}). C-terminal peptides shorter than 20-residues have not been examined. It is likely that peptides homologous to parts of the first 80 N-terminal residues of SNAP-25 would also block exocytosis. It will be appreciated that as the peptides shorten their inhibitory efficacies and avidities reduce notably ie. compare 20 and 26-mers (Ferrer-Mental *et al.*, 1998).

It is preferred that the said inhibitory SNARE may be capable of inhibiting exocytosis in a cell capable of performing SNARE-dependent exocytosis, (*in vitro* or *in vivo*), preferably a cell of the same or similar type to the said cell in a patient, by at least (in order of preference) 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 or 90% compared to a control cell to which the said inhibitory SNARE is not supplied.

The said cell (for example, cell in a patient) capable of performing SNARE-dependent exocytosis may be a nerve cell (for example a cholinergic nerve cell or an inhibitory interneurone), adreno-chromaffin cell, insulin-secreting cell (for example a pancreatic B cell), endocrine cell lines of intestinal origin (for example cholecystokinin (CCK)-secreting cells, similar to cell lines STC-1 and GLUTag; see, for example Nemoz-Gaillard *et al* (1998) *FEBS Lett* **425(1)**, 66-70) or endocrine non-intestinal cell lines (similar to, for example cell lines CA-77 and HIT-T15). Inhibition of exocytosis in a cholinergic nerve cell may be useful in producing paralysis, for example localised paralysis, in a manner similar to the use of BoNT/A for the treatment of muscular movement disorders or for cosmetic treatment, for example in which facial muscles are relaxed. Disorders which may be appropriate to treat, particularly in the field of pediatrics, are discussed in Gordon (1999) *Brain Dev* **21(3)**, 147-51 and may include strabismus and blepherospasm, spastic cerebral palsy,the extrapyramidal form of cerebral palsy, forms of dystonia, (generalized or focal), spasmodic torticollis and pain (for example back pain) caused by muscle spasms. Inhibition of exocytosis in (ie blocking of discharges from) cholinergic sympathetic and parasympathetic terminals may be beneficial, for example in the treatment of autonomic disorders, for example focal hyperhidrosis (excessive sweating).

Adreno-chromaffin cells are the secretory cells of the adrenal medulla and secrete, for example, adrenaline, the effects of which closely resemble those brought about by activity of the sympathetic nervous system. Inhibition of exocytosis from adreno-

chromaffin cells may be useful in the treatment of disorders or conditions in which excessive adrenaline release may be involved, for example stress.

It will be appreciated that the said inhibitory SNARE may be supplied to a cell that is not capable of binding to and/or taking up a clostridial toxin, for example BoNT/A *in vivo*. Thus, it will be appreciated that the said inhibitory SNARE may be useful in inhibiting exocytosis in cell types that may not at present be treated using a clostridial toxin. The said inhibitory SNARE may also be useful in inhibiting exocytosis, for example in cholinergic neurones, in patients that cannot be successfully treated using a clostridial toxin, for example BoNT/A, as a consequence of immunity to the clostridial toxin, for example as a result of previous exposure to the clostridial toxin, for example as a result of previous vaccination against botulism, for example vaccination using a BoNT/A or multivalent toxoid. It will be appreciated that patients treated with BoNT/A or other serotypes may be resistant or may develop resistance to such treatment; thus, the said inhibitory SNARE may be useful in the treatment of such patients.

For example, delivery of BoNT/B or E/ into adipocytes blocks the SNARE-dependent fusion of glucose transporter 4-containing residues with the cell surface preventing the majority of insulin-stimulated glucose uptake (i.e. control of weight gain). Chen *et al.*, 1997, Biochem. 36 p5719-5728). Thus, inhibition of exocytosis in these cells may be beneficial. It may also be appropriate to block inappropriate catecholamine secretion from adrenal chromaffin cells or pheocyromocytomas. It is likely that every cell that exhibits a regulated membrane fusion event will require SNAREs, thus abnormalities of secretion in any such cell may be potentially treatable using an inhibitory SNARE as described above.

Cell lines or cells which may be used *in vitro* to investigate the properties of an inhibitory SNARE may include adreno-chromaffin cells (see Example 1), RIN (rat) and HIT (hamster) insulin-secreting cells (discussed in Gonelle-Gispert *et al* (1999)).

De Pavia *et al* (1999) *Proc Natl Acad Sci USA* **96**, 3200-3205 describes an *in vivo* system that may be used for assessing the effect of the methods of treatment described above. The method involves repeated *in vivo* imaging of nerve terminals and measurements of nerve-muscle transmission.

A further aspect of the invention is a SNARE polypeptide for use in medicine. The SNARE polypeptide may be a native SNARE polypeptide. A further aspect of the invention provides a toxin-resistant SNARE or inhibitory or toxin-inhibitory SNARE according to any of the preceding as defined in relation to any of the preceding methods or uses of the invention for use in medicine. Preferences for the said SNARE polypeptide and the said toxin-resistant or toxin-inhibitory SNARE polypeptide and inhibitory SNARE polypeptide are as indicated above.

The above polypeptides may be made by methods well known in the art and as described below and in Example 1, for example using molecular biology methods or automated chemical peptide synthesis methods.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the

case of cysteine) and 4-methoxy-2,3,6- trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

It will be appreciated that peptidomimetic compounds may also be useful. Thus, by "polypeptide" or "peptide" we include not only molecules in which amino acid residues are joined by peptide (-CO-NH-) linkages but also molecules in which the peptide bond is reversed. Such retro-inverso peptidomimetics may be made using methods known in the art, for example such as those described in Mézière *et al* (1997) *J. Immunol.* 159, 3230-3237, incorporated herein by reference. This approach involves making pseudopeptides containing changes involving the backbone, and not orientation of side chains. Mézière *et al* (1997) show that, at least for MHC class II and T helper cell responses, these pseudopeptides are useful. Retro-inverse peptides which contain NH-CO bonds instead of CO-NH peptide bonds, are much more resistant to proteolysis.

Similarly, the peptide bond may be dispensed with altogether provided that an appropriate linker moiety which retains the spacing between the Cα atoms of the amino acid residues is used; it is particularly preferred if the linker moiety has substantially the same charge distribution and substantially the same planarity as a peptide bond.

It will be appreciated that the peptide may conveniently be blocked at its N- or C-terminus so as to help reduce susceptibility to exoproteolytic digestion.

Thus, it will be appreciated that the toxin-resistant or toxin-inhibitory SNARE polypeptide or inhibitory SNARE polypeptide, for example toxin resistant SNAP-25(Q197A/R198A or E or T) or SNAP-25(1-197), respectively, may be a peptidomimetic compound, as described above.

The SNARE polypeptide, for example toxin-resistant, toxin-inhibitory or inhibitory SNARE polypeptide, may be supplied to the cell as a molecule which comprises a further portion. It is preferred that the said further portion confers a desirable feature

on the said molecule; for example, the portion may useful in detecting or isolating the molecule, or promoting cellular uptake of the molecule or the SNARE polypeptide. The portion may be, for example, a radioactive moiety, a fluorescent moiety, for example a small fluorophore or a green fluorescent protein (GFP) fluorophore, as well known to those skilled in the art. The moiety may be an immunogenic tag, for example a Myc tag, as known to those skilled in the art or may be a lipophilic molecule or polypeptide domain that is capable of promoting cellular uptake of the molecule or the SNARE polypeptide, as known to those skilled in the art, for example as characterised for a *Drosophila* polypeptide (see, for example, Derossi *et al* (1998) *Trends Cell Biol* **8**, 84-87).

The moiety may preferably be a transport protein based on a clostridial toxin, for example as described in WO95/32738. Such a transport protein may be useful in targeting the SNARE polypeptide, for example a toxin-resistant or toxin-inhibitory SNARE polypeptide, to a cell which has been poisoned by a clostridial toxin. This may highly desirable as it may minimise side-effects on non-poisoned cells. Thus a transport protein based on a botulinum toxin may be useful in the delivery of a toxin-resistant or toxin-inhibitory SNARE polypeptide to a cell (for example a cholinergic nerve terminal) which has been poisoned, or is likely to be poisoned by a botulinum toxin, for example BoNT/A. Similarly, a transport protein based on a tetanus toxin may be useful in the delivery of a toxin-resistant or toxin-inhibitory SNARE polypeptide to a cell (for example a spinal cord cell) which has been poisoned, or is likely to be poisoned by a tetanus toxin. Details of the targeting specificities of exemplary transporter proteins are given in WO95/32738.

Thus, a further aspect of the invention provides a molecule which comprises a SNARE polypeptide or toxin-resistant or toxin-inhibitory SNARE polypeptide or inhibitory SNARE polypeptide as defined above and a further portion, preferences for which are given above. In a preferred embodiment, the invention provides a molecule

or polypeptide which comprises a SNARE polypeptide or toxin-resistant or toxin-resistant SNARE polypeptide or inhibitory SNARE polypeptide and an inactive clostridial neurotoxin having specificity (ie binding and/or uptake specificity) for a target nerve cell, for example as described in WO95/32738.

A further aspect of the invention provides a polypeptide that is a variant, fragment, derivative or fusion of SNAP-25 that is resistant to cleavage by BoNT/A or is capable of inhibiting BoNT/A (as defined above) wherein (1) the residue equivalent to residue Q197 of full length mouse SNAP-25 is replaced by A or W and the said fragment is at least 18 amino acids in length or (2) the residue equivalent to residue R198 of full length mouse SNAP-25 is replaced by H or W, and the said fragment is at least 18 amino acids in length or (3) the residue equivalent to residue Q197 of full length mouse SNAP-25 is replaced by A, K or W and the residue equivalent to R198 of full length mouse SNAP-25 is replaced by a residue other than R, for example A, T, K, H or W or (4) the residue equivalent to residue R198 of full length human SNAP-25 is replaced by a residue other than R, for example A, T, K, H or W wherein residues equivalent to one or more of amino acids 203 to 206 are not present or (5) the polypeptide is also resistant to cleavage by BoNT/E and BoNT/C or (6) the residue equivalent to residue Q197 of full length SNAP-25 is replaced by C. It is preferred that the polypeptide is also resistant to cleavage by BoNT/C1 and/or BoNT/E, as discussed above.

A further aspect of the invention provides a SNARE in which the residue immediately N-terminal to the toxin cleavage site (for example BoNT/A cleavage site) is replaced by a cysteine residue. Examples and preferences are as indicated above for a toxin-inhibitory SNARE. The said SNARE when cleaved by the clostridial toxin (for example BoNT/A in the case of SNAP-25 Q197C) may produce a potent inhibitor of the clostridial toxin, as discussed above.

It is preferred that the said variant, fragment, derivative or fusion of human SNAP-25 comprises residues identical to amino acid residues 1 to 197 of full-length human or mouse SNAP-25 or a variant thereof.

A further aspect of the invention provides a polypeptide consisting of residues identical to residues 1 to 198, 199, 200 or 201 of full length SNAP-25 or a variant thereof, or a fusion either thereof. The said polypeptide or fusion may be capable of inhibiting SNARE-dependent exocytosis, as described in Example 1 and above. It will be appreciated that the said fusion does not comprise an amino acid sequence identical to the amino acid sequence of residues 202 to 206 of full length SNAP-25 fused to the C-terminus of the polypeptide consisting of residues identical to residues 1 to 198, 199, 200 or 201 of full length SNAP-25.

A further aspect of the invention provides a nucleic acid (or polynucleotide) encoding or capable of expressing a polypeptide or molecule of the invention. A still further aspect of the invention provides a nucleic acid complementary to a nucleic acid encoding or capable of expressing a polypeptide of the invention. Methods of preparing or isolating such a nucleic acid are well known to those skilled in the art, for example as described in Example 1.

The polynucleotide or recombinant polynucleotide may be DNA or RNA, preferably DNA. The polynucleotide may or may not contain introns in the coding sequence; preferably the polynucleotide is a cDNA.

As well known to those skilled in the art, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," *Science*, **229:** 193-210 (1985), which is incorporated herein by reference.

A further aspect of the invention provides a replicable vector comprising a recombinant polynucleotide encoding a polypeptide or molecule of the invention.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to vectors for example *via* complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding the polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case of retroviral vectors, RNA) encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a

prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3, TRP1, LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda-Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic and kidney cell lines. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650 and 293 cells which are human embryonic kidney cells. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* **2**, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* **3**, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A further aspect of the invention provides a method of making a polypeptide of the invention the method comprising culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes said polypeptide, and isolating said polypeptide from said host cell. Methods of cultivating host cells and isolating recombinant proteins are well known in the art.

It will be appreciated that the above methods may also be used in relation to further polypeptides described above that are useful, for example in the methods of treatment described above, for example SNAP-25(1-197).

As indicated above, it will be appreciated that the said toxin-resistant or toxin-inhibitory SNARE or inhibitory SNARE may be supplied to the said cell by means of expression (ie synthesis) of the said toxin-resistant or toxin-inhibitory SNARE or inhibitory SNARE in the cell, for example expression of the said toxin-resistant or toxin-inhibitory SNARE or inhibitory SNARE from a recombinant polynucleotide (ie a recombinant polynucleotide capable of expressing the said SNARE) present in the cell. It will be appreciated that such supply by means of expression of the said SNARE in the target cell may be beneficial; for example, such supply may facilitate targeting of the said SNARE to the desired cell. It may also facilitate temporally-extended presence of the said SNARE or the ability to supply the said SNARE to the cell. It will be appreciated that it may be desirable to supply by means of expression both a toxin-resistant and a toxin-inhibitory SNARE to the desired cell.

Thus, a further aspect of the invention provides a recombinant polynucleotide encoding a SNARE or a variant, fragment, derivative or fusion thereof for use in medicine. The SNARE may be a toxin-resistant or toxin-inhibitory SNARE or inhibitory SNARE as defined above. The SNARE may be a polypeptide of the invention as defined above.

Suitable vectors or constructs which may be used to prepare a suitable recombinant polynucleotide may be described above.

The said SNARE may be expressed from any suitable genetic construct as is described below and delivered to the patient. Typically, the genetic construct which expresses the SNARE comprises the said SNARE coding sequence operatively linked to a promoter which can express the transcribed polynucleotide (eg mRNA) molecule in the cell, which may be translated to synthesise the SNARE polypeptide. Suitable promoters will be known to those skilled in the art, and may include promoters for ubiquitously expressed, for example housekeeping genes or for tissue-specific genes, depending upon where it is desired to express the SNARE polypeptide, as discussed further below.

Although the genetic construct can be DNA or RNA it is preferred if it is DNA.

Preferably, the genetic construct is adapted for delivery to a human cell.

Means and methods of introducing a genetic construct into a cell in an animal body are known in the art. For example, the constructs of the invention may be introduced into the cells by any convenient method, for example methods involving retroviruses, so that the construct is inserted into the genome of the (dividing) cell. Targeted retroviruses are available for use in the invention; for example, sequences conferring specific binding affinities may be engineered into pre-existing viral *env* genes (see

Miller & Vile (1995) *Faseb J.* **9**, 190-199 for a review of this and other targeted vectors for gene therapy).

It will be appreciated that retroviral methods, such as those described below, may only be suitable when the cell is a dividing cell. For example, in Kuriyama *et al* (1991) *Cell Struc. and Func.* **16**, 503-510 purified retroviruses are administered. Retroviral DNA constructs which encode said SNARE polypeptides may be made using methods well known in the art. To produce active retrovirus from such a construct it is usual to use an ecotropic psi2 packaging cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS). Transfection of the cell line is conveniently by calcium phosphate co-precipitation, and stable transformants are selected by addition of G418 to a final concentration of 1 mg/ml (assuming the retroviral construct contains a neo^R gene). Independent colonies are isolated and expanded and the culture supernatant removed, filtered through a 0.45 μm pore-size filter and stored at -70°. For the introduction of the retrovirus into the target cells, it is convenient to inject directly retroviral supernatant to which 10 μg/ml Polybrene has been added. The injection may be made into the area in which the target cells are present, for example into a muscle which it is desired to treat. It will be appreciated that retroviral delivery may be a less preferred delivery means in relation to the present invention.

Other methods involve simple delivery of the construct into the cell for expression therein either for a limited time or, following integration into the genome, for a longer time. An example of the latter approach includes liposomes (Nässander *et al* (1992) *Cancer Res.* **52**, 646-653). Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle. It will be appreciated that "naked

DNA" and DNA complexed with cationic and neutral lipids may also be useful in introducing the DNA of the invention into cells of the patient to be treated. Non-viral approaches to gene therapy are described in Ledley (1995) *Human Gene Therapy* **6**, 1129-1144. Alternative targeted delivery systems are also known such as the modified adenovirus system described in WO 94/10323 wherein, typically, the DNA is carried within the adenovirus, or adenovirus-like, particle. Michael *et al* (1995) *Gene Therapy* **2**, 660-668 describes modification of adenovirus to add a cell-selective moiety into a fibre protein. Mutant adenoviruses which replicate selectively in p53-deficient human tumour cells, such as those described in Bischoff *et al* (1996) *Science* **274**, 373-376 are also useful for delivering the genetic construct of the invention to a cell. Thus, it will be appreciated that a further aspect of the invention provides a virus or virus-like particle comprising a genetic construct of the invention. Other suitable viruses or virus-like particles include HSV, AAV, vaccinia and parvovirus.

Immunoliposomes (antibody-directed liposomes) are especially useful in targeting to cell types which over-express a cell surface protein for which antibodies are available. For the preparation of immuno-liposomes MPB-PE (N-[4-(p-maleimidophenyl)butyryl]-phosphatidylethanolamine) is synthesised according to the method of Martin & Papahadjopoulos (1982) *J. Biol. Chem.* **257**, 286-288. MPB-PE is incorporated into the liposomal bilayers to allow a covalent coupling of the antibody, or fragment thereof, to the liposomal surface. The liposome is conveniently loaded with the DNA or other genetic construct of the invention for delivery to the target cells, for example, by forming the said liposomes in a solution of the DNA or other genetic construct, followed by sequential extrusion through polycarbonate membrane filters with 0.6 μm and 0.2 μm pore size under nitrogen pressures up to 0.8 MPa. After extrusion, entrapped DNA construct is separated from free DNA construct by ultracentrifugation at 80 000 x g for 45 min. Freshly prepared MPB-PE-liposomes in deoxygenated buffer are mixed with freshly prepared antibody (or

fragment thereof) and the coupling reactions are carried out in a nitrogen atmosphere at 4°C under constant end over end rotation overnight. The immunoliposomes are separated from unconjugated antibodies by ultracentrifugation at 80 000 x g for 45 min. Immunoliposomes may be injected, for example intraperitoneally or directly into a site where the target cells are present.

Other methods of delivery include adenoviruses carrying external DNA via an antibody-polylysine bridge (see Curiel *Prog. Med. Virol.* **40**, 1-18) and transferrin-polycation conjugates as carriers (Wagner *et al* (1990) *Proc. Natl. Acad. Sci. USA* **87**, 3410-3414). In the first of these methods a polycation-antibody complex is formed with the DNA construct or other genetic construct of the invention, wherein the antibody is specific for either wild-type adenovirus or a variant adenovirus in which a new epitope has been introduced which binds the antibody. The polycation moiety binds the DNA *via* electrostatic interactions with the phosphate backbone. The adenovirus, because it contains unaltered fibre and penton proteins, is internalised into the cell and carries into the cell with it the DNA construct of the invention. It is preferred if the polycation is polylysine.

The DNA may also be delivered by adenovirus wherein it is present within the adenovirus particle, for example, as described below.

In the second of these methods, a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells is employed. This is accomplished by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, or the chicken homologue conalbumin, or combinations thereof is covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin-polycation molecules

form electrophoretically stable complexes with DNA constructs or other genetic constructs of the invention independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin-polycation and the DNA constructs or other genetic constructs of the invention are supplied to the target cells, a high level of expression from the construct in the cells is expected.

High-efficiency receptor-mediated delivery of the DNA constructs or other genetic constructs of the invention using the endosome-disruption activity of defective or chemically inactivated adenovirus particles produced by the methods of Cotten *et al* (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6094-6098 may also be used. This approach appears to rely on the fact that adenoviruses are adapted to allow release of their DNA from an endosome without passage through the lysosome, and in the presence of, for example transferrin linked to the DNA construct or other genetic construct of the invention, the construct is taken up by the cell by the same route as the adenovirus particle.

This approach has the advantages that there is no need to use complex retroviral constructs; there is no permanent modification of the genome as occurs with retroviral infection; and the targeted expression system is coupled with a targeted delivery system, thus reducing toxicity to other cell types.

It may be desirable to locally perfuse an area comprising target cells with the suitable delivery vehicle comprising the genetic construct for a period of time; additionally or alternatively the delivery vehicle or genetic construct can be injected directly into accessible areas comprising target cells. It will be appreciated that in treating a case of botulinum or tetanus poisoning, it may be beneficial to deliver the delivery vehicle or genetic construct systemically; however, it may also or alternatively be beneficial to deliver the delivery vehicle or genetic construct to the resiratory muscles as a

priority, for example by injection into the respiratory muscles, with or without more disseminated delivery, or into motor neurons in spinal cord.

The genetic constructs of the invention can be prepared using methods well known in the art.

It will be appreciated that it may be desirable to be able to regulate temporally expression of the said SNARE in the cell. Thus, it may be desirable that expression of the said SNARE is directly or indirectly (see below) under the control of a promoter that may be regulated, for example by the concentration of a small molecule that may be administered to the patient when it is desired to activate or repress (depending upon whether the small molecule effects activation or repression of the said promoter) expression of the said SNARE. It will be appreciated that this may be of particular benefit if the expression construct is stable ie capable of expressing the said SNARE (in the presence of any necessary regulatory molecules) in the said cell for a period of at least one week, one, two, three, four, five, six, eight months or one or more years. A preferred construct of the invention may comprise a regulatable promoter. Examples of regulatable promoters include those referred to in the following papers: Rivera *et al* (1999) *Proc Natl Acad Sci USA* **96(15)**, 8657-62 (control by rapamycin, an orally bioavailable drug, using two separate adenovirus or adeno-associated virus (AAV) vectors, one encoding an inducible human growth hormone (hGH) target gene, and the other a bipartite rapamycin-regulated transcription factor); Magari *et al* (1997) *J Clin Invest* **100(11)**, 2865-72 (control by rapamycin); Bueler (1999) *Biol Chem* **380(6)**, 613-22 (review of adeno-associated viral vectors); Bohl *et al* (1998) *Blood* **92(5)**, 1512-7 (control by doxycycline in adeno-associated vector); Abruzzese *et al* (1996) *J Mol Med* **74(7)**, 379-92 (reviews induction factors e.g., hormones, growth factors, cytokines, cytostatics, irradiation, heat shock and associated responsive elements). Tetracycline – inducible vectors may also be used. These are are activated by a relatively –non toxic antibiotic that has

been shown to be useful for regulating expression in mammalian cell cultures. Also, steroid-based inducers may be useful especially since the steroid receptor complex enters the nucleus where the DNA vector must be segregated prior to transcription.

This system may be further improved by regulating the expression at two levels, for example by using a tissue-specific promoter and a promoter controlled by an exogenous inducer/repressor, for example a small molecule inducer, as discussed above and known to those skilled in the art. Thus, one level of regulation may involve linking the appropriate SNAP-25 or other SNARE gene to an inducible promoter whilst a further level of regulation entails using a tissue-specific promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the SNARE gene from the inducible promoter). Control may further be improved by cell-type-specific targeting of the genetic construct. This is illustrated further (in relation to a cholinergic neuron) in Figure 9 and the legend thereto; the principle may be applied (with a suitable means of targeting to the selected cell type and suitable promoter) to other cell types.

The above system may be particularly useful when seeking localised inhibition of exocytosis through expression of an inhibitory SNARE, as described above. Techniques described in WO99/55359 (concerning nerve sprouting/nerve regeneration) may be useful. Pain in particular regions could be controlled using a promoter specific for peptidergic (eg Substance P) nerves; this would prevent synaptic transmission in sensory neurons, especially when used in conjunction with a targeting element selective for nocioceptive neurons. Expression in other neurone types may also be achieved using a promoter specific for that neurone type, as known to those skilled in the art. The cell-type specific promoter may be used directly to control the SNARE gene, or to control the gene encoding the inducible transcription factor. Further levels of control may also be used, as will be apparent to those skilled in the

art. Other examples where modulation of exocytosis may be desirable include those indicated above, modulation of the immune system, regulation of obesity by preventing insulin-stimulated glucose uptake in fat/muscle cells and blockade of spermatogenesis (as a means of contraception).

In a particularly preferred embodiment of the invention, eukaryotic expression vectors encoding, for example, a BoNT/A-resistant SNAP-25 gene and/or BoNT/A-inhibitory SNAP-25 (for example SNAP-25 Q197C) to mammalian motor nerve endings may be employed in the treatment of a patient suffering from (for example) poisoning by BoNT/A. For this purpose, a cholinergic BoNT transporter, as discussed above and described, for example, in WO95/32738 may be employed, for example by making chimeric constructs encoding the transporter and a DNA-binding protein domain, as known to those skilled in the art; the resultant hybrid protein would be complexed with the DNA constructs to be delivered, for example encoding the toxin-resistant or toxin-inhibitory SNAP-25. Alternatively, the non-toxic BoNT transporter protein could be linked to the surface of liposomal or viral delivery vehicle (after deletion of the cell binding domain) to give cholinergic specificity. Such a targeted viral-based approach may be beneficial as many non-virulent systems are comercially available, for example as discussed above, especially those that include membrane fusion elements and allow intracellular delivery of genes.

Alternatively, or in addition, neuron-specificity may be achieved by placing the genes of interest under the control of a cholinergic specific promoter (see, for example, Naciff *et al* (1999) *J. Neurochem* **72**, 17-28, which describes the identification of a 6.4-kb DNA fragment from the mouse vesicular acetylcholine transporter (VAChT) gene, encompassing 633 bp of the 5'-flanking region of the mouse vesicular acetylcholine transporter (VAChT), the entire open reading frame of the VAChT gene, contained within the first intron of the ChAT gene, and sequences upstream of the start coding sequences of the ChAT gene, which is capable of directing cholinergic

neuron-specific expression). This system may be further improved by regulating the expression at two levels, for example by using an exogenous inducer, for example a small molecule inducer, as known to those skilled in the art. Only upon addition of the low molecular weight inducer would expression of SNAP-25 occur; in this way, the time and extent of the protein's production is carefully regulated. One level of regulation may involve linking the appropriate SNAP-25 or other SNARE gene to an inducible promoter whilst a further level of regulation entails using the cholinergic promoter to drive the gene encoding the requisite inducible transcription factor (which controls expression of the SNARE gene from the inducible promoter). This is further illustrated in Figure 9 and the legend thereto.

It will be appreciated that the expressed protein must also be produced at an appropriate level relative to other synaptic proteins for optimal functioning. On some occasions higher SNARE expression maybe useful ie. the toxin-resistant SNAP-25 should be expressed in higher amounts than the BoNT/A-truncated product in BoNT-poisoned nerves (for example) so as to compete with all of the latter for binding to other SNAREs. If BoNT-resistant SNAP-25 (for example) was expressed by a cholinergic specific promotor/regulator at the level of the native cleaved product the amount produced may not cause complete rescue of exocytosis.

Thus, a further aspect of the invention provides a gene therapy delivery system comprising an inactive clostridial neurotoxin having specificity for a target nerve cell and a polynucleotide comprising a target nerve cell-specific promoter, as described above. The inactive clostridial neurotoxin may have specificity for a cholinergic neuron; it may be an inactive botulinum neurotoxin, for example an inactive botulinum toxin A, botulinum toxin B, botulinum toxin C, botulinum toxin D, botulinum toxin E, botulinum toxin F or botulinum toxin G neurotoxin. The target nerve cell-specific promoter may be specific for cholinergic neurons; it may be a promoter for vesicular acetylcholine transporter (VAChT), for example a promoter

for mouse vesicular acetylcholine transporter (VAChT), for example the promoter described above and in Naciff *et al* (1999) *J Neurochem* **72(1)**, 17-28.

It will be appreciated that the gene therapy delivery technology may be used to rescue motor nerves from toxic insults or congenital disorders, for example disorders in which inappropriate expression, membrane attachment or mutation of the sequences of SNAREs is involved. These conditions may also be treated by over- or normal expression of wild-type or the mutated SNAREs described.

It will be appreciated that the methods or constructs of the invention may be evaluated in, for example, dissociated primary neuronal cell cultures and/or nerve-muscle co-cultures, as known to those skilled in the art, before evaluation in whole animals. The methods described in de Pavia *et al* (1999) *Proc Natl Acad Sci USA* **96**, 3200-3205 may also be used in the evaluation of the methods or constructs of the invention.

The aforementioned SNAREs (for example toxin-resistant, toxin-inhibitory or inhibitory SNARE) or molecule or construct of the invention or a formulation thereof may be administered by any conventional method including oral and parenteral (eg subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time. It is preferred that the SNARE, construct or formulation is administered by injection, preferably intramuscular injection. It will be appreciated that an inducer, for example small molecule inducer as discussed above may preferably be administered orally.

Further delivery or targeting strategies may include the following. Ballistic compressed air driven DNA/protein coated nanoparticle penetration (i.e. BioRad device) of cells in culture or *in vivo* may be used. Plasmids for delivery should have cell-type specific promoters.

non-toxic (i.e. non proteolytic Light (ie L) Chain) containing heavy chain of a BoNT may be used to target and deliver thiol-containing small peptide inhibitors that are temporary oxidised (i.e. disulphide bridge) to a cysteine rich polypeptide domain added to Lchain (directly or indirectly). Once in the nerve cytosol, the reducing environment releases these once covalently linked inhibitors. Potentially many inhibitors can be delivered by a single non-toxic toxin dichain to the appropriate site of action (controlled by a number of oxidisable thiols for Light chain. Moreover, any other small molecule of clinical importance to which a thiol group can be added could potentially be delivered cholinergically.

Liposomes encapsulated SNAREs or inhibitors could be targeted by attachment of non-toxic BoNT/A dichain (attached in different ways see (4)) to their lipid surfaces in addition to fusogenic peptides (i.e. those available commercially, for example protein domains i.e. the diptheria toxin zz-domain or membrane inserted viral fusion proteins). If the low pH induced fusion properties of non-toxic BoNT/A are not sufficient when linked to liposomes to provide fusion of lipids and delivery of contents, low pH induced fusion mediating agents of the types described above may be the most useful as the cholinergic BoNT/A acceptor recycles through a low pH endosome.

There are a number of methods of non-toxic BoNT dichain attachment to liposomes or nanoparticles or viral coats. These include covalent linkage of BoNT/A via thiol directed activated liposmes i.e. MPB-PE containing liposomes *via* its 5-available free thiols. Increasing the linkage spacer may be desirable. Addition of a polypeptide domain to the N-terminus of non-active LC that enables hydrophobic membrane anchoring in an orientation that should optimise cholinergic acceptor binding domain accessibility may be useful. Addition of a His^6-tag domain (available commercially) to the BoNT dichain, preferably the Lchain N-terminus, may enable attachment to liposomes that incorporate Nickel loaded phospholipid derivatives termed DGS-NTA

(available from Avanti Polar lipids Inc) which acts as a specific ligand for His^6-tagged proteins. Use of other fusion tags i.e. MBP, GST or CaM for attachment of BoNT may be useful in concentration of ligand or loading in to liposomes during liposome loading techniques.

Whilst it is possible for a SNARE or molecule or construct of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the SNARE or the molecule or construct of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (for example, the construct or molecule of the invention) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

It will be appreciated that the SNARE or molecule or construct of the invention can be delivered to the locus by any means appropriate for localised administration of a drug. For example, a solution of the said construct can be injected directly to the site or can be delivered by infusion using an infusion pump. The construct, for example, also can be incorporated into an implantable device which when placed at the desired site, permits the construct to be released into the surrounding locus.

The construct, for example, may be administered via a hydrogel material. The hydrogel is non-inflammatory and biodegradable. Many such materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10% to about 80% by weight ethylene oxide and from about 20% to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^{R}.

In this embodiment, the hydrogel is cooled to a liquid state and the construct, for example, is admixed into the liquid to a concentration of about 1 mg nucleic acid per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the construct diffuses out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

The construct, for example, can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the construct. The construct can be incorporated into the material as it is polymerised or solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

The dose of the construct, for example, is dependent on the size of the construct and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of construct may be dependent on the size of the construct and the delivery vehicle/targeting method used and chemical composition of the oligonucleotide but a suitable dose may be determined by the skilled person, for example making use of data from the animal and *in vitro* test systems indicated above.

The construct, for example, may be administered to the patient systemically for both therapeutic and prophylactic purposes. The construct, for example may be administered by any effective method, as described above, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the construct, for example, to access and circulate in the patient's bloodstream. Construct administered systemically preferably are given in addition to locally administered construct, but also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

The invention is now described by reference to the following, non-limiting, figures and examples.

Fig. 1. Prolonged monitoring of BoNT/A- and /B-induced inhibition of catecholamine release in chromaffin cells and assessment of their SNAP-25 and Sbr/Cbr contents. Intact chromaffin cells were treated for 24 h using a LISM in the absence (open bars) or presence (hatched bars) of (A) 6.6 nM BoNT/A or (B) 66 nM BoNT/B. Cells were then maintained in culture for up to 56 days by weekly replacement of the medium. At the specified times, Ba^{2+}-evoked catecholamine release was quantified fluorometrically (means ± S.E.; n = 4). After subtraction of basal release values (cells exposed for 15 min to Locke's buffer) from the induced over 15 min by 2mM Ba^{2+} in Locke's buffer, the evoked component was expressed as a percentage of the total cell content. Immediately after measuring secretion, membrane fractions were prepared, as detailed in the Methods, solubilised in SDS and frozen at -80°C until subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies.

Fig. 2. Expression of wild-type and mutated SNAP-25 in CHO cells and assessment of cleavage by BoNT/A: subsequent evaluation of their ability to

rescue evoked secretion in BoNT/A-pre- treated chromaffin cells. (A) CHO cells, that lack SNAP-25, were transfected with the pcDNA1.1/Amp vector incorporating the specified SNAP-25 wild-type or mutated gene using Superfect reagent; after 24 h, the cells were treated with LISM in the absence or presence of 6.6 nM BoNT/A, as outlined in Experimental Procedures. Two days later, a membrane fraction was isolated from the cells and equal amounts of the SDS-solubilised proteins were subjected to SDS-PAGE and immunoblotting, using the indicated antibodies. Chromaffin cell membranes were run as a control to show that the recombinant SNAP-25 had the correct molecular weight. Western blots of relevant tracks are shown. (B) Intact chromaffin cells were treated for 24 h with LISM in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Fig. 1. After a 24 h recovery period, the cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the gene for CAT (as a control) or specified SNAP-25 variant together with the reporter plasmid encoding hGH (as outlined in the Materials & Methods section). After five days, evoked and basal hGH secretion occurring only from co-transfected cells were quantified by incubation for 15 min with a Ba^{2+}-containing and -free buffer; the hGH contents of the resultant samples were quantified using a RIA (means S.E.M.; n = 4). Values for the evoked component of secretion were expressed as a percentage of the measured total cell hGH content. Data are representative of two separate experiments.

Fig 3. Diagrammatic representation of the point-mutations generated within SNAP-25 and ELISA of their susceptibilities to proteolysis by BoNT/A. (A) Shows the structure of SNAP-25 and its C-terminal amino acid sequence, with the cleavage site of BoNT/A protease indicated. Mutations are indicated by bold letters and identified by a code name used throughout the text. (B) ELISA plate wells were coated with wild-type or specified SNAP-25 mutants before exposure for 4 h at 37°C to various concentrations of DDT-reduced BoNT/A. The amounts of substrate

remaining were probed with anti-SNAP- 25 (C-terminus)-IgG. Binding of the latter was quantified using alkaline phosphatase-conjugated anti-species antibodies and a colorimetric assay (as detailed in Materials and Methods). The resultant colour development was measured with an ELISA plate reader and absorbances at 405 nm are expressed (means ± S.D.; n > 6) as percentages of the maximal values recorded for SNAP-25-containing toxin-free wells. Symbols represent wild-type (•) or SNAP-25 mutants Q197A (O), Q197W/R198W (◇); Q197K/R198H (▼), R198A (◆), Q197A/R198A (▩), R198T (▽) and Q197A/R198K (□). Data are means (± S.D.; n ≥ 5).

Fig. 4. Exocytosis rescued by the introduction of BoNT/A-resistant SNAP-25 into BoNT/A pre-poisoned cells is inhibited by BoNT/E. Control (open bars) and 6.6 nM BoNT/A pre-treated chromaffin cells, (hatched bars and closed bar) were transfected with vectors encoding hGH and CAT or the BoNT/A resistant SNAP-25 mutant (indicated). After culturing for an additional 5 days, the transfected cells were then permeabilised using a digitonin-containing permeabilisation buffer excluding (open and hatched bars) or containing 100 nM of DTT-reduced BoNT/E (closed bar) for 15 min *(18)*. The Ca^{2+} -evoked component of hGH secretion, measured over a subsequent 15 min period, was quantified (means S.E.M; n = 4) and expressed as a percentage of the total cell content. This experiment was repeated twice, with the pattern being the same in each case.

Fig. 5. BoNT/A proteolytic activity persists in poisoned chromaffin cells for at least 3 weeks: only the protease-resistant SNAP-25 mutant rescues evoked secretion. Intact chromaffin cells were treated for 24 h with LISM , in the absence (open bars) or presence (hatched bars) of 6.6 nM BoNT/A, before being returned to growth medium for long term maintenance, as described in the legend to Fig. 1. After 16 days, the resultant cells were transfected with the pcDNA1.1/Amp expression vector containing the specified SNAP-25 or CAT gene together with the reporter

plasmid encoding hGH, as outlined before. Five days later, Ba^{2+}-evoked hGH secretion was assessed (as outlined in the legend to Fig. 2B). This experiment was repeated four times and gave the same pattern of results.

Fig. 6. Truncation and mutation of SNAP-25 to determine the C-terminal residues required for exocytosis. (A) Intact chromaffin cells were treated for 24 h with LISM in the absence (open and filled bars) or presence (hatched bars) of 6.6 nM BoNT/A, as described in the legend to Fig. 2B. The cells were then transfected with the pcDNA1.1/Amp expression vector containing either the indicated BoNT/A-resistant SNAP-25 (see panel B; open and hatched bars) or wild-type (filled bars) construct of specified length together with the reporter plasmid encoding hGH, as outlined before. After five days, Ba^{2+}-evoked hGH secretion was assessed (as outlined in the legend to Fig. 1). This experiment was repeated twice, with the pattern being the same in each case. (B) The C-terminal amino acid sequence of SNAP-25 is shown, incorporating wild-type R^{198}, or T^{198} as well any other mutations in bold letters; deletions are specified by residue numbers. Also shown is the helical wheel representation of the tetrameric SNARE complex, with the heptad positions labelled from 'a' through 'g', looking down the centre of the helix from the top at Q^{197} (arrow) and ending at L^{203}, within a representative C-terminal repeat of SNAP-25 *(27)*.

Fig. 7. Susceptibility of various SNAP-25 isoforms to BoNT/A, BoNT/C and BoNT/E (taken from Vaidyanthan *et al* (1999)). (A) Alignment of the C-terminal regions of SNAP-25, hSNAP-23 and mSNAP-23. Peptide bonds susceptible to clostridial neurotoxins are boxed. Colons (:) identify nonconservative amino acid substitutions. (B) Radiolabelled SNAP-25, hSNAP-23, and mSNAP-23 generated by in vitro transcription/translation were incubated with various recombinant botulinal L chains. Concentrations used were BoNT/A (1 nM), BoNT/C (500 nM), and BoNT/E (1 nM) for SNAP-25; 5μM each of BoNT/A, BoNT/C and BoNT/E for hSNAP-23;

and BoNT/A (5 μM), BoNT/C (5 μM) and BoNT/E (0.4 μM) for mSNAP-23. Incubation was for 60 min at 37°C for SNAP-25 or 3 h for hSNAP-23 and mSNAP-23. Cleavage was analysed by SDS-PAGE and fluorography.

Fig. 8. Amino acid sequence of human VAMP1, synaptobrevin2, mouse synaptobrevin2, human VAMP3, human SNAP-23, human SNAP-25A, human SNAP-25b and human syntaxin 1A and alignment of human and mouse SNAP-25.

Fig. 9. Gene therapy as an approach to rescuing neuro-exocytosis in botulinised patients: an example. (A) One construct, containing the cDNA for an enhancer (dark grey bar) under the control of a cholinergic promoter (cross-hatched bar) is combined with a second vector, which contains a cDNA for SNAP-25-R198T (black bar) under the control of an inducible promoter (light grey bar). These constructs are combined with DNA binding proteins (dark grey oval) and linked to a BoNT transporter (light grey mushroom) in which only the enzymatic activity was inactivated. (B) This complex, when injected locally into the muscle of bolulinsed patients, is internalised into the motor nerve endings before the DNA-protein (grey oval) complex is transported retrogradally (upward arrow) into the soma where it is taken up into the nucleus (large grey oval). After several hours of expression of the enhancer protein (black oval), a low molecular weight non-toxic inducer (dark grey circle) is added orally. Addition of the inducer to the enhancer allows expression of BoNT/A-resistant SNAP-25 (dark striped bar), as shown in (A). This soluble protein becomes membrane bound upon palmitoylation at the Golgi (open oval). This membrane-bound SNAP-25-R198T is trafficked anterogradally (downward arrow) to the endplate where the high levels of BoNT/A-resistant SNAP-25 overcomes the blockade of transmission.

Example 1. Rescue of Exocytosis in Botulinum Toxin A-Poisoned Chromaffin Cells by Expression of Cleavage-Resistant SNAP-25: Identification of its Essential C-terminal Residues

Botulinum neurotoxin (BoNT) types A and B selectively block transmitter release by cleavage of SNAP-25 and synaptobrevin, respectively; in humans, many months are required for full recovery from the resultant neuromuscular paralysis. To decipher the molecular basis for such prolonged poisoning, intoxication in adreno-chromaffin cells was monitored over 2 months. Exocytosis in BoNT/B-treated cells resumed after 56 days due to the appearance of intact synaptobrevin. However, inhibition continued in BoNT/A-treated cells, over the same interval, due to the persistence of cleaved SNAP-25 (1-197). When recovery of exocytosis was attempted by transfection of poisoned cells with the gene encoding full-length SNAP-25 (1-206), no restoration of exocytosis ensued even 3 weeks post intoxication. To ascertain if this failure was due to the persistence of the toxin's protease activity, the cells were transfected with constructs encoding BoNT/A-resistant mutated form of SNAP-25; importantly, expression of these mutants rescued exocytosis. Stepwise trunctation of the toxin-insensitive SNAP-25 at the C-terminus revealed that residues 1-201, 1-202, 1-203 afforded a significant return of exocytosis, unlike shorter forms 1-197, -198, -199 or -200 which proved inhibitory; accordingly, mutants M202A or L203A of full-length SNAP-25 rescued secretion. These collective findings give further insights into the functional domain of SNAP-25, demonstrate the longevity of BoNT/A protease and provides the prospect of a therapy for botulism.

The abbreviations used are: BoNT/A, /B, /C and /E, botutinum neurotoxins type A, B, C and E; BSA, bovine serum albumin; CAT, chloramphenicol-acetyl transferase; Cbr, cellubrevin; CHO, Chinese hamster ovary; DTT, dithiothreitol; GST, glutathione-S-transferase; hGH, human growth hormone; IgG, immunoglobulin type G; LC, light chain; LDCV, large dense-core vesicle; LISM low-ionic strength

medium; NMJ, neuromuscular junction; NSF, N-ethylmaleimide sensitive fusion protein; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RIA. radio-immunoassay; Sbr, synaptobrevin; SNAP-25, synaptosomal-associated protein of Mr = 25 kDa; SNAP-25_A (1-197) and SNAP-25_C (1-198), the products of proteolysis by BoNT/A and BoNT/C, respectively; SNARE, soluble NSF attachment protein receptor; SDS, sodium dodecyl sulphate; TeTX tetanus toxin.

Botulinum neurotoxin type A (BoNT/A) is successfully employed for the therapy of dystonias and dysphonias because it potently and selectively inhibits acetylcholine release at the neuromuscular junction (NMJ) that results in paralysis lasting for several months (1,2) (and reviewed in (3)). Seven serotypes of BoNTs (A - G) produced by *Clostridium botulinum* whereas only a single form of neurotoxin, tetanus toxin (TeTX) is synthesised by *Clostridium tetani;* the latter blocks transmitter release at central inhibitory nerve terminals (4). These homologous but immunologically distinct proteins share many properties; each active neurotoxin consists of a heavy chain (HC) and a light chain (LC) linked by a disulphide and non-covalent bonds. The HC is required for high affinity binding to specific neuronal ecto-receptors, subsequent internalisation and translocation into the cytosol, where the LC blocks synaptic-vesicle exocytosis [reviewed by (4)]. The LCs of BoNTs and TeTX are Zn^{2+}-dependent endoproteinases that exbibit remarkable substrate selectivities, targeting single bonds (except for BoNT/C, see below) in one of three proteins proposed to constitute the core components (SNAREs) of the vesicle docking/fusion complex which mediates the regulated exocytosis of neurotransmitters (5). Synaptosomal-associated protein of Mr = 25 kDa (SNAP-25) is proteolysed by BoNT/A, /C and /E at separate sites near the C-terminus: Q^{197}-R^{198}, R^{198}-A^{199}, and R^{180}-I^{181} (6-8), respectively. Syntaxin 1A/B is cleaved by BoNT/C and synaptobrevin (Sbr) by BoNT/B, /D, /F, /G and TeTX [reviewed by (4)]. Unlike syntaxin 1A/B and Sbr which possess a transmembrane spanning region (9,10), SNAP-25 is linked to the membrane through thioester-linked palmitate modifications at one or more of its four

centrally-located cysteines (11). Whilst cleavage of syntaxinl or Sbr by the appropriate Clostridial neurotoxin inhibits exocytosis through the truncation of the domain required for ternary complex formation (12,13), BoNT/A or E truncated SNAP-25 inhibits release without preventing ternary complex formation (12). BoNT/A- or E-truncated SNAP-25 remain membrane-bound, but release is inhibited due to destabilisation of the ternary complex even though assembly and disassembly can still occur (Hayashi, T *et al* (1994) *EMBO J.* 13, 5051-5061; Pellegrini, L. L *et al*(1995) *EMBO J.* 14, 4705-4713). The precise basis of their blockade of neuroexocytosis has yet to be elucidated.

Whilst the mechanism of BoNT-induced inhibition has received much attention, the molecular processes involved in recovery of the NMJ from poisoning, which can take several months depending on the serotype used, remains poorly understood. In humans, the level of reversal of neuromuscular paralysis with either BoNT/A- or /B was found to be ~10% and ~70%, complete at 49 days post-injection (2). Recently, the sequence of steps in this eventual resumption of neurotransmission in poisoned motor end-plates has been revealed by monitoring synaptic function in identified poisoned nerve endings of living animals (14). These studies showed that the transient appearance of functional nerve sprouts mediates a partial return of nerve-induced muscle twitch, with full recovery relying on the originally-poisoned nerve terminals re-acquiring synaptic activity. However, it still remains unclear if and how the turnover of the SNAREs affects the recovery process.

In order to gain insights into the molecular basis of this extended recovery period, which seems to vary with the serotype used, a similar study was undertaken in adreno-chromaffin cells. Evoked exocytotis from the latter shares many characteristics with synaptic vesicle-mediated neurotransmitter release in neurons; in particular, SNAP-25, Sbr and syntaxin 1 are present and have all been shown to be essential for large dense-core vesicle (LDCV) release [for review see (15)] because their cleavage by

BoNT/A, /B or /C blocks exocytosis in chromaffin cells. As previous work on these cells had suggested that persistent BoNT/A or TeTX activity inhibited catecholamine release for prolonged periods (16), it was pertinent to compare the time course of recovery of exocytosis in chromaffin cells to the different profiles of recovery from poisoning of NMJ in humans with BoNT/A and B (2). This model cell system allowed the life-time of the respective BoNT-truncated SNARE to be assessed directly, a question that cannot be easily answered at the NMJ. Further, it was hoped to overcome BoNT/A-poisoning in chromaffin calls through the introduction of vectors expressing wild-type SNAP-25. The failure to rescue exocytosis in this way provided proof for the continued activity of the toxin for 3 weeks. The successful production of SNAP-25 mutants that are highly resistant to BoNT/A but capable of mediating exocytosis when transfected into the cells allowed rescue from poisoning to be achieved, This novel outcome also demonstrated that substituting the residues in SNAP-25 at positions 197, 198, 202 or 203, ie including those comprising the BoNT/A susceptible bond, does not affect regulated secretion whereas truncating SNAP-25 past residue 201 or 202 inhibited the process.

Experimental procedures

Materials- Highly purified BoNT/A was isolated as previously described (17). BoNT/B and /E were supplied by Drs C. C. Shone (CAMR, Porton Down, Salisbury) and B. DasGupta (Madison, Wisconsin, USA), and activated prior to use (18). Antiserum specific for SNAP-25 (-197) was a gift from Drs T.A.N. Ekong and D. Sesardic (NIBSC, Herts, UK). Mouse cDNA encoding SNAP-25b and a BoNT/A-truncated version were provided by Dr L. Zhou of this laboratory. Urografin (Schering Healthcare, G.D.R.), digitonin (Novabiochem U.K.), Radio-immuno-assay (RIA) kit (Nichols Institute, San Capistrano, CA, USA), QuickChange™ (Stratagene, Netherlands), calcium phosphate reagents (GIBCO-BRL, Scotland, UK), Superfect™

(Qiagen, UK), pGEX-2T and ECL reagents (Amersham Pharmacia, UK) and pcDNA 1.1 (Invitrogen, The Netherlands) were purchased.

Culture of cells and intoxication with BoNT/A or /B - Bovine chromaffin cells were prepared from bovine adrenal glands and maintained as primary cultures, as described previously (19). Cells required for transfection were further enriched (to remove any contaminating fibroblasts) using Urografin density-gradient centrifugation, as previously detailed (20). Chinese hamster ovary (CHO) cells, which lack SNAP-25, were plated at 50-70% confluency on 6-well plates. Within 2-3 days after preparation, cells were incubated with a low ionic strength medium (LISM) at 37°C for 24 h in the absence or presence of 6.6 nM BoNT/A or 66 nM BoNT/B and maintained at 37°C for up to 56 days by weekly replacement of medium (19).

Stimulation and quantification of catecholamine secretion from intact chromaffin cells - Immediately before measuring release, cells pre-treated with or without BoNT/A or /B were briefly washed with Locke's solution (20) and then incubated in quadruplicate at 22°C with this buffer without additions, or containing 2 mM Ba^{2+}. After 15 min, aliquots of the medium bathing the cells wen removed and assayed for catecholamine content, using a fluorometric procedure (19). Values for basal release of catecholamine were subtracted from the respective evoked components and expressed as a percentage of the total contents.

Expression of wild-type or mutant SNAP-25 in cells: sole monitoring of transfected cells using a human growth hormone (hGH) reporter system - At the indicated times after incubation in LISM with or without 6.6 nM BoNT/A, chromaffin cells were transfected with the hGH construct together with pcDNA1.1/Amp-CAT or the appropriate SNAP-25 gene, using the calcium phosphate precipitation method (21). Four to six days after transfection, hGH secretion from intact chromaffin cells was stimulated and quantified as described for catecholamine release but using a RIA.

Some transfected cells were permeabilised with 20 μM digitonin in a permeabilisation buffer (18) and exposed for 15 min to reduced BoNT/E. Evoked release was assessed over a subsequent 15 min by the addition of 20 μM free Ca^{2+}. Aliquots were removed and assayed for hGH content. Ca^{2+}-evoked hGH secretion was calculated as described above.

Generation of mutated and C-terminally-deleted SNAP-25 genes by PCR-based mutagenesis techniques – Site-specific point or stop mutations (listed in Fig. 2A and 6A) were introduced into the wild-type (wt) SNAP-25b mouse gene using *Pfu*-DNA polymerase by either one- or two-step PCR amplification or Quick-Change™ site-directed mutagenesis. This construct had previously been subcloned into pGEX-2T or pcDNA1.1/Amp using the *Bam*H1 and *Eco*R1 restriction sites. For the one- or two-step PCR amplification procedure, the forward and reverse primers incorporated *Bam*H1 and *Eco*RI sites, respectively. The SNAP-25 C-terminal deletion mutant, residues 1-198 (also termed SNAP-25c), was generated using the forward and reverse primers 5'-AGA CGG ATC CAT GGC CGA GGA CGC-3'/ 5'-AGC ATG AAT TCT CAA CGT TGG TTG GCT TCA-3', respectively, and religated into the pGEX-2T vector. Alternatively, several of the SNAP-25 double-point mutants (Q197A/R198A, Q197A/Rl98K Q197W/Rl98W) were generated using the specified forward primer and the following reverse primers 5'-CAT CTT TGT TGC AGC TGC GTT GGC TTC AT-3', 5'-CAT CTT TGT TGC CTT TGC GTT GGC TTC CAT-3' or 5'-AGC ATC TTT GTT GCC CAC CAG TTG GCT TCA TC-3', respectively, and were religated into the vector. All the remaining mutations (R198T, Q197A, R198A and Q197K/R198K) were generated using the *Dpn*-l nuclease Quick-Change procedure. The respective forward and reverse primer pairs used were: 5'-GAT GAA GCC AAC CAA ACT GCA ACA AAG ATG CTG GG-3' / 5'-CCC AGC ATC TTT GTT GCA GTT TGG TTG GCT TAT C-3', 5'-GAT GAA GCC AAC GCA CGT GCA ACA AAG-3' /'5-CCC AGC ATC TTT GTT GCA CGT GCG TTG GCT TCA TC-3', 5-GAT GAA GCC AAC CAA GCT GCA ACA AAG ATG CTG GG-3' / '5-

CCC AGC ATC TTT GTT GCA GCT TGG TTG GCT TCA TC-3' and '5-CCA GAA TTG ATG AAG CCA ACA AAC ATG CAA CAA AGA TGC TG-3' / '5-CAG CAT CTT TGT TGC ATG TTT GTT GGC TTC ATC AAT TCT GG-3'. The remaining C-terminal deletion mutants [1-199(R198T), 1-200(R198T) and 1-202(R198T)] were generated using the Quick-Change procedure and pcDNA1.1/Amp-SNAP-25-RI98T as the template. The respective forward and reverse primer pairs used were: '5-GAT GAA GCC AAC CAA ACT GCA TAA AAG ATG CTG GGA AGT GGT-3' / '5-ACC ACT TCC CAG CAT CTT TTA TGC AGT TTG GTT GGC TTC ATC-3', 5'-GAT GAA GCC AAC CAA ACT GCA ACA TAG ATG CTG GGA AGT GGT-3'/ 5'-ACC ACT TCC CAG CAT CTA TGT TGC AGT TTG GTT GGC TTC ATC-3' and 5'-GCC AAC CAA CGT GCA ACA AAG ATG TAG GGA AGT GGT-3' / 5'-ACC ACT TCC CTA CAT CTT TGT TGC ACG TTG GTT GGC-3'. The various mutations in the SNAP-25 gene were verified by sequencing.

Assessment of susceptibility of bacterially-expressed wild-type and mutant SNAP-25 to BoNT/A cleavage using a novel ELISA - Full-length variants of glutathione-S-transferase (GST)-SNAP-25 were expressed in E. coli and purified by affinity chromatography (Smith, D. B., and Johnson, K. S. (1988) *Gene* **67**, 31-40). Each of the recombinant proteins was coated onto 96-well plates, rinsed, blocked with 2% ($^{W}/_{V}$) bovine serum albumin (BSA) and exposed to various concentrations of DTT-reduced BoNT/A at 37 C for 4 h. The wells were then aspirated, washed and probed with anti-SNAP-25 (C-terminus)-IgG (generated against the last 12 residues of SNAP-25) (19). Unbound IgG was removed by rapid washing and the primary IgGs bound were detected indirectly using anti-species-specific IgGs covalently conjugated to alkaline phosphatase with measurement of the colour developed upon addition of para-nitrophenyl phosphate. The A405nm values recorded from toxin-treated wells were expressed as percentages of those for toxin-free control and plotted against the toxin concentration used. Standard curves relating amounts of intact SNAP-25 remaining in wells were made using defined mixtures of full-length and BoNT/A-truncated GST-

SNAP-25. The A405nm readings observed were expressed as percentages of that recorded for the 100% intact protein sample and plotted against the amounts of intact GST-SNAP-25 coated initially. Generally, ~60-70 % of the maximum A405nm signal represented 50 % intact SNAP-25 in walls.

Assessment of the proteolytic activities of BoNT/A or /B in cell cultures - CHO cells, which lack SNAP-25, were transfected with pcDNA1.1/Amp-SNAP-25s using Superfect and 24h later were incubated in LISM with and without 6.6 nM BoNT/A, as described for chromaffin cells. Membranes were isolated, subjected to immunoblotting with the following IgGs (19,23); anti-SNAP25(recombinant)-IgG (an antibody raised against full-length recombinant GST-SNAP-25), anti-SNAP-25_A (an antiserum solely reactive with SNAP-25_A), anti-SNAP-25(C-terminus)-IgG (an antibody generated against the last 12 residues of SNAP-25) and anti-Sbr/Cbr-IgG (an antibody generated against residues 33-94 of human Sbr-2). Bound antibodies were detected indirectly using anti-species-specific IgGs covalently conjugated to horseradish peroxidase and visualised by the enhanced chemiluminesence (ECL)-detection system.

Results

Recovery of evoked secretion from poisoning with BoNT/A and B shows different time courses: a process coincident with reappearance of intact SNAREs

To investigate the important question of how BoNTs exert their inhibitory actions for prolonged periods neuroendocrine chromaffin cells were intoxicated for 24 h with BoNT/A (6.6 nM) or B (66 nM using LISM to overcome the absence of high afffinitv acceptors (19). Evoked secretion and contents of SNAREs were assessed at 5, 19, 40 and 56 days post intoxication (Fig. 1). Ba^{2+} was employed instead of other common stimuli (e.g. 55 mM K^+ or nicotine that induce Ca^{2+} entry) because it evokes much more catecholamine release (up to 50% of the total content compared to only ~20 % by the latter stimuli) while still exhibiting the same requirements for SNAP-25 and

Sbr/Cbr as that induced by Ca^{2+} (19). As expected, BoNT/A or /B treatments for 5 days yielded extensive inhibition of Ba^{2+}-evoked catecholamine release of 84 ± 0.70% and 90 ± 0.4% (means ± S.E.; n = 4), respectively, relative to that for toxin-free controls. Intact SNAP-25 was monitored using anti-SNAP-2.5(C-terminus)-IgG, whilst intact Sbr and Cbr (a close BoNT-sensitive homologue (24)) were quantified using anti-Sbr/CbrIgG. Both of these antibody preparations were non-reactive towards the BoNT cleaved products (the membrane-retained portion of the antigens). Further, the presence of BoNT/A-truncated SNAP-25 (in the membrane) was monitored using IgG specific for $SNAP\text{-}25_A$. This antibody was unreactive with intact SNAP-25 in neurotoxin-free cells, but reacted strongly with the truncated product in BoNT/A poisoned cells (Fig. 1; and as found previously (18)). Western blotting of the requisite cells' membrane fraction showed that the BoNT-mediated inhibition of secretion noted at day 5 was accompanied by total or near-complete cleavage of intact SNAP-25 or Sbr/Cbr (Fig. 1); as expected, the BoNT/A truncated SNAP-25 was readily detectable at this time (Fig. 1A). At day 40 post-intoxication, a trace of intact SNAP-25 was observed in BoNT/A-poisoned cells; however, the amount noted was much lower than normal and insufficient to significantly influence the level of evoked secretion recorded compared to day 5 (Fig. 1A). However, at day 56 post-intoxication, slightly larger amounts of intact SNAP-25 were noted in the poisoned cells, apparently enough to cause a statistically significant level of recovery of exocytosis (from 84 to 66% inhibition; from 16 to 34% control). Throughout this time course, $SNAP\text{-}25_A$ persisted in the intoxicated cells (Fig. 1A). Difficulties inherent to primary cell cultures precluded studies longer than 8 weeks post-intoxication, periods that would appear to be necessary to attain complete recovery from BoNT/A poisoning.

Conversely, a much faster time course was observed for virtually complete recovery from blockade by BoNT/B. The inhibition of evoked secretion in BoNT/B poisoned cells noted on day 5 of 90 ± 0.4%. gradually subsided until only 10 ± 3.4%

inhibition (means ± S.E.; n = 4) remained at day 56. Return of evoked secretion in BoNT/B-treated cells was coincident with reappearance of intact Sbr/Cbr to a level comparable to that observed in toxin-free cells (Fig. 1B).

SNAP-25 expression demonstrated in CHO cells: appraisal of the ability of wild-type to rescue evoked exocytosis in BoNT/A-pre-treated chromaffin cells. Based on the above-noted findings, it was hypothesised that expression of an excess of the full-length SNAP-25 in BoNT/A-poisoned cells might restore regulated exocytosis by competing with the SNAP-25_A present. As a prerequisite to testing this possibility, expression of SNAP-25 in mammalian cells had to be demonstrated; this could be most readily achieved by transfecting pcDNA1.1/Amp-SNAP-25 into CHO cells that lack endogenous SNAP-25. SDS-PAGE and Western blotting of a Triton X-100 solublilised extract of the cells showed that SNAP-25 was expressed and exhibited the same Mr as the native species present in chromaffin cells (Fig. 2A), when probed with anti-SNAP-25(full-length)IgG; it was also detectable with anti-SNAP-25(C-terminus)-IgG but not by IgG specific for SNAP-25_A (Fig. 2A). Notably, exposure of these transfected cells to BoNT/A resulted in the loss of labelling with anti-SNAP-25(C-terminus)-IgG and the appearance of SNAP-25_A reactivity (Fig. 2A), establishing the susceptibility of the recombinant protein to the toxin.

To evaluate the prospect of rescuing release through the introduction of SNAP-25 into BoNT/A-poisoned chromaffin cells, the protein was transiently co-expressed with hGH. The latter, which is normally absent from these cells, co-localises with catecholamines in LDCVs; thus, evoked hGH secretion serves as an excellent reporter for LDCV exocytosis (21) . Control cells transfected with vectors encoding hGH and the non-toxic protein, CAT, exhibited an appreciable level of Ba^{2+}-evoked hGH release, and this was inhibited extensively in cells pre-treated with BoNT/A (Fig. 2B). Notably, these two values approximate to the respective levels of stimulated catecholamine secretion observed for non-transfected cells (Fig. 1A). Transfection

with a plasmid encoding wild-type SNAP-25 did not alter or increased slightly the amount of hGH secreted from toxin-free cells; significantly, such over-expression of SNAP-25 failed to overcome the extent of inhibition due to BoNT/A poisoning (Fig. 2B). These results suggested that the persistence of BoNT/A protease for 5 days after intoxication maintained the blockade of release; to exclude other factors, SNAP-25 mutants insensitive to BoNT/A were constructed and tested.

Generation of mutant SNAP-25 highly resistant to proteolysis by BoNT/A

We embarked on the preparation of forms of full-length SNAP-25 that were not susceptible to BoNT/A but might be able to mediate exocytosis. Amino acids at positions 197 and/or 198 in SNAP-25 located on either side of the cleaved bond (i.e. the P1 or P'1 sites - nomenclature of (25)) were altered, since earlier studies (26) found that the P'1 arginine residue (equivalent to residue 198) is critical for cleavage of SNAP-25 peptides by BoNT/A. Thus, appropriate nucleotide bases in the SNAP-25b gene were altered by PCR-based site-directed mutagenesis and mutant proteins expressed as GST-linked products (see Experimental Procedures). Seven different SNAP-25 mutants were generated containing either single or double substitutions at the P1 and/or P'1 positions (listed in Fig. 3A). Each GST-SNAP-25 variant was isolated/purified by affinity chromatography; SDS-PAGE and Western blotting confirmed that they exhibited the appropriate mobilities and immunoreactivities (not shown). For measurement of proteolysis of recombinant SNAP-25 by BoNT/A, we optimized and standardised an ELISA which allows quantitation of the amount of full-length SNAP-25 remaining. Notably, wildtype and SNAP-25 mutants all exhibited different susceptibilities to BoNT/A (Fig. 3B); the EC_{50} values for the cleavage of each were extrapolated from Fig. 3B and displayed in Table 1. These results revealed that changes at the P'1, but not at the P1, position yielded proteins highly resistant to BoNT/A compared to the wild-type substrate; replacement of P'1 arginine with either alanine (R198A) or threonine (R198T) reduced degradation of SNAP-25 by ~550- and ~16,000-fold respectively (Table 1). In contrast, alteration of the P1 glutamine to

alanine caused little change (Q197A; Table 1). On the other hand, double point mutations incorporating the innocuous P1 alanine replacement in addition to alterations at the P'1 site to alanine (Q197A/R198A) or lysine (Q197A/R198K) greatly decreased sensitivity to proteolysis; by type A protease, with Q197A/R198K providing the highest degree of resistance (38,000-fold reduction in susceptibility). Other double-point mutations at the P1 and P'1 positions [i.e. the Q197/R198 sequence to either KH [naturally-occurring in *Torpedo* SNAP-25 (27) or WW] caused far less resistance to BoNT/A (~440- and 96- fold, respectively; Table 1). It was hoped that at least some of these SNAP-25 variants generated could rescue evoked exocytosis from inhibition by BoNT/A.

TABLE 1: The overall susceptibilities of wild-type and various SNAP-25 mutants to cleavage by BoNT/A

SNAP-25 Variant	Position of mutated residue(s)	Minimum BoNT/A concentration (nM) necessary to proteolyse 50% of SNAP-25 in the standard assay[a]	Susceptibilities to proteolysis by BoNT/A (relative to wild-type)[b]
Wild-type Q197/R198	None	0.0045	1.0
Q197A	P1	0.0054	1.2
R198A	P1`	2.5	550
R198T	P1`	72	16000
Q197A/R198A	P1, P1`	20	4400
Q197A/R198K	P1, P1`	170	38000
Q197K/R198H	P1, P1`	2.0	440
Q197W/R198W	P1, P1`	0.43	96

a Standard curves, relating increasing amounts of intact GST-SNAP-25 per well to the increasing A405 nm values recorded, were used to calculate the A405nm reading which was equivalent to 50% intact SNAP-25 remaining in wells. This absorbance value was used to extrapolate the concentrations of BoNT/A required to proteolyse 50% of either the wild-type or mutant SNAP-25 from Fig. 3B.

b Values obtained by dividing the minimal BoNT/A concentration giving 50% cleavage of SNAP-25 mutant by the minimal concentration which cleaves wild-type; larger values indicate greater resistance to proteolysis.

For the initial rescue experiments, R198T was selected for several reasons: (i) its high resistance to BoNT/A; (ii) a single substitution was deemed more likely than a double to be able to mediate exocytosis, and cause rescue in toxin-treated cells and (iii) in the unlikely event of it being cleaved intracellularly, the product would be detectable with anti-SNAP-25_A-IgG because of the retention of Q^{197} (which was altered in the double mutants). As a preliminary to the complicated rescue experiments, the expression of R198T and its lack of susceptibility to BoNT/A were first established in CHO cells because they lack endogenous SNAP-25. After transfection, as described above, R198T was found to be expressed at a similar level to wild-type SNAP-25, as judged from the labelling on blots using two antibodies reactive with full-length SNAP-25 or its C-terminal region (Fig. 2A). Anti-SNAP-25(recombinant)-IgG, which recognizes both the full-length and BoNT/A-truncated form, detected expression of SNAP-25 in cells transfected with the wild-type, mutant R198T or SNAP-25_A mammalian expression constructs (Fig. 2). Moreover, recombinant SNAP-25 proteins exhibited the appropriate Mr compared to the native species present in chromaffin cells (Fig. 2). Exposure of the transfected cells to BoNT/A did not lower the R198T reactivity with anti-SNAP-25(C-terminus)-IgG and, furthermore, no product could be observed with SNAP-25_A-IgG (Fig. 2A). Having demonstrated that R198T mutant was expressed, but not cleaved by BoNT/A in mammalian cells, plasmids encoding several such SNAP-25 mutants were introduced into chromaffin cells.

Notably, both the wild-type and R198T proteins from toxin-free cells showed equivalent reactivities with antiSNAP-25(C-terminus)-IgG; instead, SNAP-25_A was solely recognised by IgG specific for SNAP-25_{1-197} (Fig. 2). In addition, the expressed SNAP-25s exhibited equivalent sensitivities to BoNT/A protease as the proteins generated in *E. coli* (not shown). Exposure of transfected cells to BoNT/A resulted in the disappearance of wild-type but not R198T immunoreactivity when probed with anti-SNAP-25 (C-terminus)-IgG. Importantly, SNAP-25_A reactivity was observed in

BoNT/A-treated cells transfected with wild-type but not R198T (Fig. 2). As expected, immunoreactivity of SNAP-25$_A$ was unaffected by BoNT/A treatment (Fig. 2). Having demonstrated that R198T mutant was not cleaved by BoNT/A in mammalian cells, plasmids encoding wild-type and mutant SNAP-25 were introduced into chromaffin cells.

Effects of expressing SNAP-25$_A$ and protease-resistant mutants on evoked exocytosis from control or BoNT/A-pretreated chromaffin cells

It was important to determine whether the expressed SNAP-25 participates in exocytosis. In control or BoNT/A treated cells that had been transfected with vectors encoding hGH and the non-toxic protein CAT, similar amounts of Ba^{2+}-evoked hGH and catecholamine secretion were observed (Fig. 1, 2B). Importantly, expression of the BoNT/A-cleaved product, SNAP-25$_A$, in toxin-free cells inhibited evoked-secretions of hGH by >90%, in a manner similar to BoNT/A pre-treatment. Moreover, expression of SNAP-25$_A$ in BoNT/A pre-poisoned cells consistently abolished the residual evoked secretion (~10 % of total) that commonly remained following toxin treatment (Fig. 6A). Thus, not only does introduced SNAP-25 replace the endogenous protein, the displacement of the intact SNAP-25 with the BoNT/A-truncated product establishes that BoNT/A poisoning is due solely to cleavage of SNAP-25.

Having validated the system, transfection of plasmids directing the expression of BoNT/A protease-resistant SNAP-25s was performed. Introduction of those mutants into toxin-free chromaffin cells caused only minimal reductions in abilities to secrete hGH, as compared to controls (transfected with wild-type SNAP-25 or an unrelated protein which is not involved in exocytosis (CAT)) (Fig. 2B). As hoped, expression of each protease-resistant SNAP-25 in BoNT/A-treated cells (in which catecholamine release is inhibited; data not shown) almost completely rescued secretory function, close to the levels recorded for toxin-free controls (Fig. 2B). This experiment was

repeated twice, with the same outcome; clearly, expression of BoNT/A protease-resistant SNAP-25s can efficiently rescue regulated secretion in cells poisoned 5 days earlier.

Additional experiments were undertaken to demonstrate conclusively that the rescue of evoked hGH secretion in BoNT/A pre-intoxicated cells was a direct result of the participation of expressed BoNT/A protease-resistant SNAP-25. Digitonin-permeabilisation provided a means to introduce BoNT/E into cells (because the LISM-based protocol is ineffective for this serotype) so that it could proteolyse the expressed BoNT/A protease-resistant R198T mutant and, thereby, negate its protective effect. As expected in cells lacking protease-resistant SNAP-25, prior poisoning with BoNT/A caused an 82 $\pm$4.3% inhibition of Ca^{2+}-evoked hGH release relative to toxin-free CAT control. On the other hand, evoked hGH secretion in BoNT/A pre-intoxicated cells expressing the protease-resistant R198T mutant was largely protected. Importantly, when BoNT/A-pre-treated cells expressing R198T mutant were permeabilised and exposed to reduced BoNT/E (using conditions known to proteolyse nearly all the SNAP-25 (18)), the above-noted restoration of evoked secretion was largely abolished and replaced with an 88$\pm$7.8% inhibition of evoked hGH secretion (Fig. 4). (This experiment was repeated twice, with the pattern being the same in each case).

Three weeks after exposure to BoNT/A, only protease-resistant SNAP-25 rescues regulated exocytosis: indirect demonstration of the presence of active toxin protease

The ability to rescue exocytosis upon transfection of a mutated but not wild-type SNAP-25 in BoNT/A poisoned cells suggested that the toxin's activity persisted for at least a week (Fig. 2B). To determine how long BoNT/A remained active, this experiment was repeated with cells exposed for a longer period to BoNT/A (Fig. 5). As expected, toxin-free cells transfected with hGH and CAT encoding control

plasmids (i.e. recombinant SNAP-25-free control) released large amounts of hGH in a divalent cation-dependent manner (Fig. 5). In contrast transfection of cells exposed to BoNT/A 3 weeks previously, with the same vectors resulted in much reduced amounts of evoked secretion (Fig. 5). Therefore, toxin pre-treatment still afforded a large extent of blockade after 21 days. As before, expression of wild-type SNAP-25 did not significantly alter the amounts of evoked hGH secretion occurring in toxin-free cells and failed to overcome the blockade (Fig. 5). Hence, it seems likely that the newly-expressed wild-type SNAP-25 was cleaved by BoNT/A protein remaining active in the cells. Accordingly, the extent of evoked hGH secretion recorded from cells intoxicated 3 weeks earlier when expressing protease-resistant SNAP-25 R19ST was comparable to that occurring from toxin-free cells transfected with either the same mutant or CAT (see above; Fig. 5). This experiment was repeated four times and gave the same pattern of results.

Identification of the minimal C-terminal amino acids of SNAP-25 necessary for exocytosis *in situ*

$SNAP\text{-}25_A$, which lacks the last 9 C-terminal residues (198-206), inhibits exocytosis when expressed (Fig. 6A) or produced by BoNT/A cleavage of full-length SNAP-25 (Fig. 1A). Moreover, its expression in BoNT/A-prepoisoned cells consistently diminished the residual evoked secretion of hGH that commonly remained following toxin treatment (Fig. 6A). Thus, it appears that the endogenous intact SNAP-25 can be displaced from the SNARE complex by $SNAP\text{-}25_A$; hence, this product mediates the inhibition of exocytosis by BoNT/A. Thus, it was pertinent to determine how many of the C-terminal residues were redundant for exocytosis, by generating constructs that allowed sequential trunctation of SNAP-25. For assessment of recovery upon introduction of these constructs into BoNT/A-treated cells, it was necessary to use SNAP-25 deletion constructs resistant to BoNT/A (Figure 6B). Recent structural data on the SNAP-25 C-terminal domain (Poirier, M. A *et al* (1998) *Nat. Struct. Biol.* **5**, 765-769) suggests that when it participates in the core complex

residues 141-206 form a helical structure in which one complete turn involves seven residues, labelled 'a' to 'g'(Fig. 6B). The identification of the maximum number of residues that can be removed from the C-terminal end of SNAP-25, without affecting its ability to rescue exocytosis, would indicate where this functional domain terminates. Wild-type, full-sized protease-resistant R198T and SNAP-25_A mutants were employed in this set of experiments to define the highest and lowest extents of secretion. The four C-terminally deleted mutants generated encompassed residues 1-198, 1-199, 1-200 and 1-202; the latter three additionally contained the R198T replacement in order to render it profoundly protease-resistant; 1-198 [analogous to the product resulting from BoNT/C proteolysis, termed SNAP-25c, see example (8)] also proved highly resistant to type A because of the extensive deletion (data not shown). In this regard, it is noteworthy that the expression of SNAP-25_{1-198} [the product of cleavage with BoNT/C1 and known to be resistant to BoNT/A], SNAP-25_{1-199}-R198T or SNAP-25_{1-200}-R198T inhibited secretion in toxin-free or -treated cells (Fig. 6A) relative to that seen with full-length SNAP-25-R198T (Fig. 6A). In contrast, a normal level of secretion was observed with SNAP-25_{1-202}-R198T or SNAP-25_{1-203} -R198T in toxin-free cells; moreover, all of these BoNT/A-resistant mutants rescued release in BoNT/A pre-poisoned cells (Fig. 6A). The noted ability of SNAP-25_{1-201}-R198T to support exocytosis to a significant but lower level than SNAP-25_{1-206}-R198T suggested that the R198T mutation might compromise slightly the ability of SNAP-25 to function in exocytosis which only becomes apparent in this shortened construct. Indeed, this seems to be the case because the wild-type R^{198} version of SNAP-25_{1-201} gave the control level of release, when introduced into non-poisoned chromaffin cells. To conclusively prove that residues 197 to 201 are sufficient for normal exocytosis, the adjoining M^{202} was mutated to an alanine in full-length SNAP-25, significantly, SNAP-25_{1-206}-M202A preserved the full complement of hGH secretion (Fig 6A). Further, expression of the BoNT/A-resistant SNAP-25_{1-206}-R198T/M202A not only supported exocytosis in

non-intoxicated cells but it rescued secretion in poisoned chromaffin cells (Fig. 6A). Thus, it seems that wild-type SNAP-25 encompassing residues 197-201 can mediate exocytosis without M^{202} or the last 4 residues. As a recent study (Criado, M *et al* (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7256-7261) indicated that mutating L^{203} to alanine in wild-type SNAP-25 suppressed exocytosis in chromaffin cells, this point mutant was evaluated. Introduction of SNAP-25_{1-206}-L203A into chromaffin cells caused no inhibition of hGH secretion (Fig 6A); further, expression of its BoNT/A-resistant variant, SNAP-25_{1-206}- R198T/L203A, mediated exocytosis in non-intoxicated cells and afforded near-complete rescue of secretion after poisoning (Fig. 6A). This discrepancy may relate to the use of SNAP-25 mutants tagged with Green Fluorescent Protein (GFP) in the other study.

Discussion

The different recovery periods for regulated exocytosis in BoNT/A- and B- poisoned chromaffin cells reported herein accords with published data for both chromaffin cells (16) and that found after focal intramuscular injection in humans (2). Also, it is shown in this study that the near-complete recovery observed after intoxication of chromaffin cell with BoNT/B is due to the re-appearance of intact Sbr/Cbr. It is significant that the time required for this correlates with that found for chromaffin cells poisoned with TeTX (16) suggesting that either the turnover of BoNT/B and TeTX are very similar and/or the rate of replenishment of intact Sbr/Cbr determines the speed of recovery. The much slower rates for recovery, reappearance of newly-synthesised intact SNAP-25, and disappearance of the truncated product in BoNT/A-treated cells show that either this serotype remains active for longer than BoNT/B and/or the turnover of SNAP-25 is significantly slower than that of Sbr/Cbr. Whilst SNAP-25 synthesis has been detected in chromaffin cells (28), its content is 200-fold lower than that found in synaptic terminals (29). However, we show for the first time that BoNT/A is proteolytically active for 3 weeks after poisoning, because recovery was observed when toxin resistant mutants but not wild-type SNAP-25 were

overexpressed in BoNT/A poisoned cells. This indicates that the toxin cleaved all of the wild-type SNAP-25 produced in the transfected cells. Such persistent activity suggests that, at least in chromaffin cells, the continued action of BoNT/A-LC is a major contributory factor in the prolonged inhibition of release. Moreover, our data indicates that toxic amounts of active BoNT/A protein may remain in cells even 56 days post-exposure and continue to do so until the eventual complete recovery of evoked secretory function (not determined). Whilst this agrees with previous findings on chromaffin cells (16), it may seem to contradict some recent data. Human muscles double poisoned by injection with a mixture of BoNT/A and /E recovered as if they had been solely intoxicated with BoNT/E, which shows a shorter timecourse for recovery than A (30). These results suggest it is not the continued BoNT/A activity but the persistence of SNAP-25_A that limits recovery. The removal of 17 extra amino acids from the C-terminal end of SNAP-25_A may lead to a more rapid recovery of neurotransmission because the shorter SNAP-25 fragment is less stable in the cells and/or is less inhibitory, for example may form a less stable complex. The duration of poisoning, which is similar in both of these models, is determined by the ratio between the amount of toxin activity persisting and the rate of SNAP-25 synthesis; although available information suggests that this ratio is lower in motor nerve endings than chromaffin cells; slow recycling of SNAP-25_A in the nerve terminals (suggested by the accelerating effect of BoNT/E) could prolong the recovery period proposed in Eleopra, R *et al* (1998) *Neurosci. Lett.* **256**, 135-138.

Constructs were prepared that produced SNAP-25 forms resistant to BoNT/A but still capable of supporting exocytosis. Our findings establish that the P'1 residue of SNAP-25 is very important for defining efficient cleavage of full-length SNAP-25 by BoNT/A, which agrees with that found using smaller C-terminal 17-mer substrates (residues 187-203) of SNAP-25 (26). During the course of this study, others (8) reported a similar high resistance of SNAP-25 to scission by BoNT/A when P'1 replacements were incorporated.

Significantly, it is shown herein for first time, that introduction of these BoNT/A-resistant forms of SNAP-25 into intact chromaffin cells rescues exocytosis; this successful "*in vivo*" recovery system provided some important insights about SNAP-25 and its BoNT/A cleavage site. First, over-expression of either wild-type SNAP-25 or BoNT/A-resistant mutants does not seem to cause toxicity to the cell, since the extent of evoked hGH release from toxin-free cells appears to be unaffected. In addition, recombinant SNAP-25 has been previously shown to be targeted to the appropriate plasma membrane locations in PC12, as well as insulinoma calls (31,32). As SNAP-25 must complex with the limited amounts of syntaxin 1 and Sbr present in order to exert its co-operative exocytotic function, it is reasonable to assume that most of the excess protease-resistant SNAP-25s competitively displaces native SNAP-25 from SNARE complex formation. The fact that BoNT/B inhibits this rescue of exocytosis provides further proof for the participation of BoNT/A-resistant SNAP-25 in SNARE-mediated exocytosis. In light of these important considerations, it is evident that residues Q197 and R198 in SNAP-25, which were altered in the protease-resistant mutants, are not essential for evoked exocytosis. Our own studies and those of others provide mounting evidence for there being a high degree of amino acid degeneracy in SNAP-25, when the BoNT/E cleavage site (residues 180-181) in SNAP-25 was mutated, it was found that over-expression of BoNT/E-resistant SNAP-25 prevented the anticipated inhibition of evoked secretion upon exposure to BONT/E (32), which suggests that these residues are also not critical for exocytosis. Further, a recent comprehensive site-directed mutagenesis study of SNAP-25 has revealed that mutations of "a" and/or "d" positions (Fig. 6B), which are buried hydrophobic residues within the core of the four-helix bundle, lowered the stability of SNAP-25:syntaxin 1A/B:Sbr ternary complexes and diminished the ability of this region of SNAP-25 to perform its function in regulated vesicular fusion (33). The full extent of this degeneracy was seen when human SNAP-23 (a BoNT/E-resistant non-neuronal homologue which is only ~60% identical to SNAP-25) was over-expressed in

permeabilised insulinoma cells; it supported the same extent of exocytosis upon treatment with BoNT/E as that found for control cells (34). However, the present study is the first reported study in which the amino acid degeneracy of SNAP-25 has been shown through the ability of BoNT/A-resistant mutants to rescue exocytosis in intact pre-poisoned cells.

As the expression system adopted here enabled accurate evaluation of the relative functionality of mutated and wild-type SNAP-25, it was deemed important to determine the minimal requirement for exocytosis of amino acids located at position 198 to 206 (ie. those excised by BoNT/A). This is an important issue because recent structural data on SNAP-25 show that the C-terminal core domain orientates in such a way that it appears to make the initial contact with Sbr/Cbr (Poirier *et al* (1998) *Nat. Struct. Biol.* **5** , 765-769). Previous *in vitro* studies have shown that removal of this 9-residue section from the C-terminus of SNAP-25 largely diminished its ability to form binary complexes with Sbr but not with syntaxinl (35,36). Although another study (12) found no reduction in the ability of SNAP-25_A to form this ternary complex it did report that the resulting complex was less stable as indicated by its lowered resistance to SDS- and elevated temperature-mediated dissociation. This latter finding is also supported by other work (13). In comparison, binary complexes of Sbr and syntaxinl or SNAP-25 exhibited only weak affinities (12,35). In support of the possibility that BoNT/A cleavage of SNAP-25 does not prevent ternary associations (or vesicle docking), over-expression of SNAP-25_A in insulinoma cells (37) caused accumulation of significantly higher numbers of secretory vesicles proximal to the plasma membrane (relative to control cells). Whilst SNAP-25_A may be able to form a ternary complex, the extensive inhibition of secretion observed could be due to its inability to participate in the vesicle-plasmalemma fusion mechanism. Thus, the C-terminal fragment released might itself be inhibitory to the fusion mechanism. For example, it has been shown that a 20-mer or a 26-mer C-terminal peptide of SNAP-25 both inhibited release in permeabilised chromaffin cells with half-maximal inhibitory

effect requiring between 10 to 20 μM peptide (36), although the nine amino acid peptide spanning residues 198-206 was not tested.

The SNAP-25 deletion mutant study performed herein has shown that not all of the residues encompassing positions 198-206 are required for the participation of SNAP-25 in exocytosis. In particular, removal of four amino acids from the C-terminal end of SNAP-25, incorporating the BoNT/A resistant R198T alteration, rescued evoked secretion in BoNT/A poisoned cells as efficiently as the full-length mutant. Further residues 198 to 200 are essential for exocytosis, since they failed to rescue exocytosis. The lack of rescue observed upon introduction of SNAP-25 (1-198) is significant since it shows that the product generated by BoNT/C proteolysis does not support exocytosis. This results was expected but had not been proven because of the dual action of BoNT/C, which proteolyses both syntaxinlA/B and SNAP-25 (20,25,37). Thus, the truncation of SNAP-25 by BoNT/C is inhibitory in its own right regardless of its cleavage of syntaxinlA/B. It is also noteworthy that residue 199, in the C-terminal predicted helical domain of SNAP-25 which is removed by BoNT/C, has been shown to come in close contact with the predicted helical domains of N-terminal SNAP-25 and syntaxin 1A/B (38). However, our studies show that the four amino acids at the C-terminal of SNAP-25 are redundant for its normal functioning. The deletions studied herein established that only residues encompassing positions 1-201 are absolutely essential for the participation of SNAP-25 in exocytosis, with residues 1-200 failing to rescue release; and furthermore, SNAP-25 lacking the last five amino acids without the BoNT/A resistant R198T alteration, proved as effective as the full-length mutant rescuing evoked secretion in BoNT/A poisoned cells. Further evidence was provided for 202 to 206 being redundant by demonstrating that point mutations at positions 202 or 203 did not affect their ability to support secretion. During the preparation of this work, another study showed that only three of these C-terminal amino acids are redundant for SNAP-25 functioning (39). This apparent discrepancy could be explained in one of several ways. First, a slower rise time for exocytosis

cells expressing SNAP-25(1-202) might explain why a complete inhibition of exocytosis was only observed within 10 seconds but a partial inhibition within 1 minute in the latter study, compared with slight inhibition being apparent within 15 minutes in our experiments (Fig. 6A). Second, the mutation at R198T might render the coil-coil domain more stable, making L^{203} redundant for regulated exocytosis. However, amino acids at the "g" position, such as R198, tend to be charged; changing this amino acid might cause a reduction in the ability of the C-terminal end of SNAP-25 to interact with syntaxin 1 (Fig. 6B), which is consistent with our data (Fig. 2B, 4, 5 and 6A). Third, the use of a GFP tag at the N-terminal end of SNAP-25 in the other investigation might have slightly altered its ability to support exocytosis when this truncation was introduced. A scenario that would reconcile these apparently different findings would imply that there is not a single cut off point which determines its ability to support exocytosis. Our work (Fig. 6A) and theirs suggest that the addition of each of 3 residues after 200 improves exocytosis to a small extent until the maximum is reached with 203. Thus, the differences between the abilities of constructs 1-201, 1-202 or 1-203 to support exocytosis is slight.

Previous work from this lab (18) demonstrated that intact cells can be poisoned with BoNT/A such that virtually all of their SNAP-25 present has been truncated and near-complete inhibition of exocytosis achieved. Yet, when the same cells were permeabilised with digitonin, a significant amount of release could be obtained upon Ca^{2+}-stimulation, equivalent to ~50 % of the response from non-intoxicated cells. Importantly, this BoNT/A-resistant release from permeabilised cells was dramatically lowered by subsequent addition of BoNT/E. Similar attenuation of the exocytotic block induced by BoNT/A has been observed when cytosolic Ca^{2+} concentrations are abnormally elevated in both BoNT/A-poisoned motor nerve endings (42) and cerebrocortical synaptosomes (43). There might be a pool of LDCV at the plasma membrane, in which SNAP-25 is protected from BoNT/A, by SNARE complex formation. The BoNT/A-resistant response is largely dependent on MgATP which is

required to disassemble SNARE complexes during priming (44). However, this does not explain the lack of response from intact cells exposed to a depolarising stimuli. The BoNT/A-resistant release could be mediated by SNAP-23, a homologue of SNAP-25 that is cleaved by BoNT/E but not A (34). However, the trace of SNAP-23 that exists in primary cultures of chromaffin cells (28) is present only in contaminating fibroblasts (data not shown). Finally, SNAP-25 might play two roles; initially intact SNAP-25 is required for tethering of the LDCV to the membrane. Subsequently, removal of 26 amino acids ($SNAP\text{-}25_E$) but not 9 amino acids ($SNAP\text{-}25_A$) prevents fusion from taking place (18). The latter two scenarios would explain why over-expression of $SNAP\text{-}25_A$ causes a more profound inhibition when compared to cells exposed to BoNT/A (Fig. 4A and (41)), that is, over-expressed SNAP-25 displaces all the endogenous SNAP-25 homologues independent of their activation state. The fact that this partial reversal after BoNT/A poisoning was observed with catecholamine release (data not shown and (18)) but not with hGH (Fig. 4B), suggests that the BoNT/A resistant pool of LDCVs is not being recycled, Thus, these hGH-free LDCVs retain their ability to undergo fusion.

The majority of the outbreaks of human botulism are caused by intoxication with BoNT/A, /B or /E, (reviewed in (45)), and, at present the only useful treatment relies on the prompt administration of neutralising antibodies. Unfortunately, this treatment is ineffective once the toxins are internalised inside motor nerves and patients start displaying symptoms of botulism. Although effective toxoids exist for vaccination, they are increasingly unpopular because of the widespread and successful use of BoNTs in the treatment of numerous muscle movement disorders. In view of this recently acquired attitude and the above-noted factors, then is a requirement to design novel treatments for botulism. One such approach that is being pursued is the development of small peptide-based inhibitors (26). An alternate and attractive approach involves, for example, the use of gene therapy in which neurotransmitter release is rescued by expression of BoNT/A protease-resistant SNAP-25 in motor

neurons. Our work clearly demonstrates that the regulated exocytotic pathway is not required for the transfer of SNAP-25 to the plasma membrane and shows that the constitutive pathway, which transfers palmitoylated SNAP-25 to the plasma membrane from the Golgi (46), is not blocked in chromaffin cells by BoNT/A. Thus, this supports a gene therapy-based approach.

In summary, the following points should be emphasised. First, in chromaffin cells BoNT/A-LC appears to remain active for at least three weeks, as introduction of wild-type SNAP-25 does not rescue release, unlike toxin-resistant forms. Second, BoNT/A poisoning can be rescued through the introduction of BoNT/A-resistant SNAP-25. Finally, the last four C-terminal amino acids (residues 202 to 206) of SNAP-25 appear to be redundant for its functioning in exocytosis. Residue 201 may also be redundant for its functioning in exocytosis.

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Example 2: rescue of exocytotic fuction in BoNT/A-poisoned cells by introduction of an expression vector containing a protease-resistant SANP-25 mutant.

Rescue of exocytotic function in BoNT/A-poisoned cells is achieved by introduction of an expression vector containing a gene encoding a SNAP-25 mutant, shown to be resistant to proteolytic cleavage by this toxin. We demonstrate in a neuroendocrine model system - adreno-chromaffin cells - that inhibition of Ca^{2+}-dependent exocytosis by exposure to BoNT/A was evident after 56 days. The near-maximal level of blockade (about 90%) observed initially was accompanied by an abolition of intact SNAP-25, as revealed by Western blotting with antibodies raised against the C-terminal residues removed by BoNT/A. Even two months after the intoxication, there was minimal reappearance in these cells of intact SNAP-25 but, instead, virtually all of the protein remained in the truncated form and, thus, detectable using IgG reactive exclusively with the cleaved product. Accordingly, evoked catecholamine release remained inhibited after 8 weeks by 70% relative to that in non-toxin treated samples.

Such prolonged poisoning may have arisen from a slow removal of the truncated target and this, in its own right, might inhibit the functioning of the full length SNAP-25. This possibility was examined by artificially introducing genes encoding one or other of these two protein forms into bovine chromaffin cells, using a mammalian expression vector (pcDNA1-1/amp). After insertion of the coding sequences for wild-type or BoNT/A-truncated SNAP-25 (1-197) into the latter, successful expression of the requisite proteins in CHO calls was demonstrated by Western blotting. In order to evaluate the effects of these expressed proteins on evoked exocytosis it was necessary to co-transfect chromaffin cells with either of these two constructs, and the same vector containing the gene for human growth hormone (hGH) to allow monitoring of secretion only from the transfected cells (Holz *et al* (1995) *Meth. Enzymol* **257**, 221-231). hGH, normally absent from chromaffin cells, is targetted to the regulatory secretory pathway into the dense-core vesicles that contain catecholamines and, thus, serves as an excellent reporter for exocytosis from transfected cells, when quantified by a sensitive radio-immunoassay. Chromaffin cells cotransfected in this way with wild-type SNAP-25 and hGH gave a high level of Ba^{2+}-evoked exocytosis, matching that seen in cells transfected with a control plasmid encoding an irrelevant, non-toxic protein, CAT (Fig. 2). In notable contrast this secretion was virtually abolished from cells transfected likewise but with $SNAP25_{1-197}$. Presumably, the observed inhibition results from the expressed truncated protein competing with the smaller amounts of endogenous SNAP-25 for binding to the limited pool of native syntaxin 1 and synaptobrovin (or homologues) present, excluding full-length SNAP-25 from the ternary complex required for exocytosis. Our results establish that an excess of $SNAP\text{-}25_{1-197}$ over the native form, persisting in BoNT/A-treated cells as noted above,

could antagonise the functioning of minimal quantities of resynthesised SNAP-25 that escape cleavage.

This proven technology to express full-length SNAP-25 was then used in an attempt to rescue exocytosis in cells pre-exposed to BoNT/A. The toxin treatment caused near-complete inhibition (~ 94%) of evoked hGH secretion in cells cotransfected with CAT and hGH; the blockade was not restored upon transfection of SNAP-25 and hGH (Fig. 2). As this clear-cut outcome highlighted the possibility of the toxin's protease remaining active over several days, it was necessary to test constructs for mutants of SNAP-25 found to be non-susceptible to cleavage by BoNT/A. With the aim of obtaining such variants which would retain ability to mediate neuro-exocytosis, minimal alterations were made in full-length SNAP-25 at positions 197 and/or 198 which are located on either side (P1, P'1) of the bond cleaved by BoNT/A (Fig. 3 and 5). These mutated constructs were expressed in *E. coli* as fusion proteins with glutathione-S-tranferase, affinity purified and assessed for cleavage by BoNT/A, using an ELISA. The resultant recombinant proteins all exhibited decreased susceptibility to the toxin, but to different levels relative to the wild-type SNAP-25 (Table 1). Most showed extraordinarily high resistance to BoNT/A in this protease assay, a result confirmed by their successful expression in CHO cells and the subsequently observed lack of cleavage by intracellularly applied BoNT/A (compared to wild type which became readily truncated). These data established the suitability of such mutants for potential rescue of BoNT poisoning, and confirm the findings of Dr J Schmidt and colleagues (Schmidt and Bostian (1997) *J Prot Chem* **16**, 19-26) with short SNAP-25 peptides which demonstrated that P'1 arginine is crucial for efficient cleavage. Co-transfection of chromaffin cells with hGH and wild-type SNAP-25 or mutants gave near-equivalent amounts of Ba^{2+}-evoked secretion (Fig. 2 and 5), confirming that none of the single or double mutations affected their ability to mediate regulated exocytosis. Most importantly, expression of any of five BoNT/A-resistant mutants of

SNAP-25 rescued exocytosis; strikingly, this was complete or very substantial (Fig. 5). As the recovery could not be achieved by expression of wild-type SNAP-25, it can be concluded that this was due to the continued action of the toxin's protease. These novel findings establish the proof of principle for relatively fast rescue (over a few days) of BoNT/A intoxication. Encouraged by this success, the length of time that the BoNT activity can survive was assessed by transfecting chromaffin calls that had been poisoned with BoNT/A three weeks previously. Again expression of the protease-resistant mutant, R198T, but not wild-type SNAP-25 rescued secretion completely. This is a conclusive demonstration that an unusually long-term persistence of the protease activity of BoNT/A is a major contributing factor in the extended duration of this poisoning. In support of the validity of the chromaffin cell model, the recovery time from neuromuscular paralysis following injection of patients with BoNT/A in a clinical trial (Sloop *et al* (1997) *Neurology* **49**, 189-194) is remarkably similar to that observed with these cells; furthermore, the shorter duration of action of BoNT/B in the latter study corresponds to the faster return of exocytosis noted for chromaffin cells treated with this serotype.